Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 49. MARCH, 1942. No. 3.

SECTION MEETINGS

CLEVELAND	
Western Reserve University	February 13, 1942
DISTRICT OF COLUMBIA	
Georgetown University	February 15, 1942
IOWA	
State University of Iowa	February 17, 1942
MINNESOTA	
University of Minnesota	February 18, 1942
PACIFIC COAST	
University of California	February 4, 1942
ROCKY MOUNTAIN	
University of Colorado	February 21, 1942
WESTERN NEW YORK	
University of Rochester	February 14, 1942

13549

Cure of Repeated Attacks of Nutritional Muscular Dystrophy in the Rabbit by Alpha-Tocopherol.*

C. G. MACKENZIE. (Introduced by E. V. McCollum.)

From the Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md.

The extreme degeneration of the skeletal muscles in rabbits originally described in detail by Goettsch and Pappenheimer[1] was sub-

* Supported by grants from the Research Corporation of New York and the Carnegie Institution of Washington.

The technical assistance of Miriam Reed and Jeanette Nickles is gratefully acknowledged.

1 Goettsch, M., and Pappenheimer, A. M., *J. Exp. Med.*, 1931, **54**, 145.

sequently shown by us to be the result of vitamin E deficiency.[2] At that time we reported the absence of microscopic abnormalities in the muscles of rabbits cured of severe dystrophy with vitamin E. In a later experiment 3 successive attacks of the disease were produced and cured.[3] Goettsch and Pappenheimer[4] have recently described the very rapid restoration of normal muscle tissue in dystrophic guinea pigs following vitamin E therapy.

The present investigation was undertaken to determine whether or not the remarkable reparative process elicited by α-tocopherol would persist after a long series of attacks, and to throw some light, if possible, on the rather discouraging results obtained by many workers in the treatment of human dystrophies with α-tocopherol. Undoubtedly chronic and intermittent deficiencies in the human diet are much more common than persistent complete deficiencies.

Seven young male and female rabbits weighing from 340 to 640 g each were placed on dystrophy-producing diet 13.[1] The criteria employed to diagnose dystrophy; namely, creatinuria, loss of weight, reduced food consumption, and physical symptoms, have been previously discussed in detail.[2] After stage II dystrophy (rabbits easily laid on their sides) or, in the great majority of cases, stage III dystrophy (rabbits readily pushed off their feet) had persisted for several days, the animals were cured with a single dose of d, l-α-tocopherol† in ethyl laurate solution. This procedure was repeated during each attack of the disease. Five to 15 mg of α-tocopherol were used to cure the first 3 attacks, and 20 to 40 mg of the vitamin for subsequent attacks.

From 4 to 6 attacks of severe dystrophy were thus produced and cured in experimental periods of from 16 to 32 weeks. As the number of attacks increased, the disease tended to run a somewhat longer course before reaching a dangerously acute stage. Two typical growth curves are shown in Fig. 1. The response to α-tocopherol was typical[2, 5] in all cases with the exception of the final attack in one animal to be described below. The high urinary creatine promptly fell to a normal level of 10 mg or less per day,

[2] Mackenzie, C. G., and McCollum, E. V., *J. Nutrition*, 1940, **19**, 345.

[3] Mackenzie, C. G., Levine, M. D., and McCollum, E. V., *J. Nutrition*, 1940, **20**, 399.

[4] Goettsch, M., and Pappenheimer, A. M., *J. Nutrition*, 1941, **21**, Proc. Am. Inst. Nutr., p. 7.

† Supplied by Merck and Company, Inc.

[5] Mackenzie, C. G., and McCollum, E. V., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 642.

growth was resumed, appetite restored, and the physical symptoms disappeared. There was no decline in the rapidity of response to vitamin E with successive attacks, and the duration of the growth response following each treatment was roughly proportional to the dose of α-tocopherol administered.

Rabbits Nos. 218 and 220 were allowed to succumb to their 4th and 5th attacks of dystrophy at 23 and 19 weeks respectively. Rabbit No. 222 died with diarrhea during its 5th attack 2 days after receiving α-tocopherol. The remaining 4 animals were subjected to 4, 5, or 6 attacks of the disease, and were then given 10 mg of α-tocopherol daily until they were sacrificed 2 to 4 weeks later. The response to this comparatively high level of α-tocopherol was excellent with one exception. Although vitamin E therapy produced a prompt drop in the creatine excretion of rabbit No. 225 from a level of 45 to 20 mg daily, and an immediate growth response as shown in Fig. 1, the daily creatine excretion still averaged 20 mg after 3 weeks of treatment. The α-tocopherol was then increased to 20 mg daily, and the creatine fell to 10 mg in 3 days. In all of the other attacks the daily creatine excretion fell to 10 mg or less 4 or 5 days after therapy was initiated.

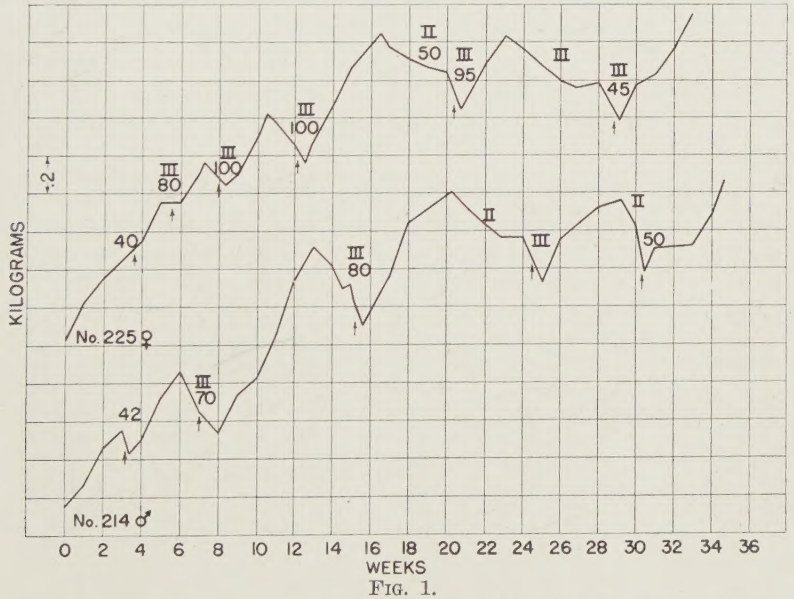


FIG. 1.

Growth curves of young rabbits subjected to repeated attacks of dystrophy. The administration of α-tocopherol is indicated by arrows. The daily creatine excretion and the stages of dystrophy observed during the attacks are shown by the Arabic and Roman numerals above the growth curves.

Sections were prepared from the vastus lateralis, biceps femoris, and ventricles of the hearts of all of the animals, and in the case of the males from the epididymides and testes. All sections were stained with hematoxylin and eosin, and in addition those from the thigh muscles were stained with Van Gieson's connective tissue stain. The thigh muscles from the 3 rabbits dying with dystrophy showed severe hyalinization and necrosis, and an increase in cellular elements as described by Goettsch and Pappenheimer.[1] Connective tissue and fat were definitely increased in the muscle sections taken from these animals. On the other hand, the thigh muscles of the 4 rabbits treated with α-tocopherol for from 2 to 4 weeks (following their last attack of dystrophy) were devoid of hyalinization and necrosis, and contained no accumulations of cellular elements. While the amounts of connective tissue and fat were apparently less than those observed in the muscles of the 3 dystrophic rabbits, this may have been due entirely to a relatively greater volume of muscle tissue in the cured animals, for the former elements were still readily discernible. The number of attacks of dystrophy, the final treatment, and the degree of muscle hyalinization and necrosis are given in Table I.

Histological examination of the ventricles revealed no myocardial lesions with the exception of several accumulations of cells with large, pale, elliptical or irregular nuclei in the left ventricle of 2 animals (Nos. 218 and 220) dying with dystrophy. Morphologically these lesions resemble those described by Miller[6] in normal rabbits.

Five of the 7 animals, including the 3 dying with dystrophy, were males. The epididymides of these 5 rabbits contained an abundance of sperm, and very few germ cells (no more than are commonly

TABLE I.

Effect of Alpha-tocopherol on Muscle Lesions in Dystrophic Rabbits Previously Subjected to Repeated Attacks of the Disease.

Rabbit No.	Sex	Initial wt, kg	No. of attacks	Final attack, wks	Final wt, kg	Final treatment	Microscopic muscle lesions
218	♂	.34	4	23	1.5	Died	+++
220	♂	.60	5	19	1.7	"	+++
222	♂	.62	5	16	2.2	"	+++
227	♂	.56	6	32	2.4	α-t* 2 wks	—
215	♀	.36	5	31	2.3	α-t 3 "	—
214	♂	.35	5	31	2.1	α-t 4 "	—
225	♀	.64	6	29	2.4	α-t 4 "	—

*d,l,-α-tocopherol.

[6] Miller, C. P., *J. Exp. Med.*, 1924, **40**, 543.

found in our normal animals). Although not abundant, sperm were found in many tubules of the testes of all 5 rabbits. They were more plentiful in the cured animals than in those dying with dystrophy. Aside from a slight sloughing of germ cells in the testes of rabbit No. 218, which died with dystrophy, and in the testes of rabbit No. 227, which was cured of the disease, the germinal epithelium of all animals was normal. This confirms and extends our recent finding[7] that widespread muscle lesions occur in vitamin E-deficient rabbits in the absence of testicular degeneration.

In this experiment the dystrophic rabbits developed no symptoms other than those referable to changes in the skeletal muscles. Furthermore, the remarkable stimulus given to muscle repair by α-tocopherol therapy was not lost even after 6 attacks of the disease.

Conclusions. As many as 6 successive attacks of nutritional muscular dystrophy have been produced and cured in rabbits. Continued α-tocopherol therapy following the last attack resulted in the complete repair of hyalinization and necrosis of the thigh muscles. Testicular degeneration was not observed.

13550 P

Production of Subcutaneous Sarcoma by Azo Dye and the Influence Thereon of Liver Feeding.

JOSEPH C. TURNER,* AND BARBARA MULLIKEN. (Introduced by W. W. Palmer.)

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.

Interest in the liver tumors produced by azo dyes has recently been directed chiefly along two lines, to wit: (a) studies of the inhibition of carcinogenesis by accessory food substances, particularly the demonstration that extracts of liver or yeast will protect rats against the butter-yellow liver cancer,[1] and (b) the apparent difference in biological activity between the azo compounds and the polynuclear hydrocarbons which might be dependent upon the presence

7 Mackenzie, C. G., and McCollum, E. V., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 148.

* This work was carried out in part under a fellowship grant from the Commonwealth Fund.

1 Sugiura, K., and Rhoads, C. P., *Cancer Research*, 1941, **1**, 3.

or absence of nitrogen in the molecule.[2] " . . . . the hydrocarbons usually produce tumors at the site of application whereas all of the above nitrogen-compounds except styryl 430 tend to exert an effect at a remote site." " . . . . it might be helpful even at this early stage to learn if the presence of nitrogen in the majority of compounds which act at a distant site is significant or merely a coincidence."[2] Observations made in this laboratory during the past few months would appear to bear both upon the problem of the inhibition of carcinogenesis by food supplements and upon the relation of nitrogen content to carcinogenic activity. We have found that the nitrogenous compound *o*-aminoazotoluene may in fact produce tumors at the site of injection as well as in the liver, and moreover that the development of such local tumors, in contrast to hepatomas, appears not to be inhibited by the addition of liver to the diet.

Liver cancer may be induced in rats or mice by the incorporation of *p*-dimethylaminoazobenzene (butter yellow) or *o*-aminoazotoluene in the food, and by subcutaneous injection of these dyes dissolved in olive oil or suspended in glycerin.[3] The development of local tumors at the site of application has not been reported.

Our studies were begun in December, 1940, for the purpose of procuring a liver cell carcinoma with which to carry out certain immunological investigations. Sixty young adult mice of the highly inbred C strain were given *o*-aminoazotoluene in a course of injections extending now more than a year. The mice belonged to a family bred in this laboratory from pedigreed C strain parents obtained from the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Me. Males and females were employed in equal number. Every mouse received 2.5 mg of dye weekly until the termination of the experiment. The dye was dissolved in corn-oil ("Mazola") and injected subcutaneously into the back through a No. 24 needle, 0.1 cc of oil being used for each dose. Forty of the animals were maintained on a stock diet of Purina chow pellets, while the remaining 20 were given a supplement of dried fresh beef liver, 20% by weight added to powdered Purina pellets and finally made into small cakes by moistening with water and drying.

Almost a year after the beginning of the experiment, tumors began to appear in some of the mice. Meanwhile about half of the animals, both of liver-fed and control groups, had died, presumably from the toxicity of the carcinogen. This report is based upon

[2] Fieser, L. F., *Cause and Growth of Cancer*, University of Pennsylvania Press, 1941.

[3] Shear, M. J., *Am. J. Cancer*, 1937, **29**, 269.

TABLE I.
Tumors in Treated C Strain Mice.

	Controls Total No., 7*		Liver fed Total No., 3
Interscapular tumor	5 (71%)		3 (100%)
Normal liver	1		2
Cirrhotic "	3		0
Cirrhosis and adenoma	1	71%	1
Carcinoma	1		0

*One liver specimen lost.

the findings in 10 mice brought to autopsy during November and December of 1941, some 11 months after the beginning of the experiment. At the present writing, 22 additional mice are still under treatment; these will be reported upon at a later date.

Results are shown in Table I. Subcutaneous sarcomata† developed in 8 of the 10 mice, and these were found invariably in the interscapular region, a site at which blebs of injected oil may often be seen. Spontaneous subcutaneous sarcomas are found very rarely in the C strain mouse.[4]

While the feeding of liver protected against liver cirrhosis and cancer, it does not appear to influence the development of local sarcomata since these tumors developed in all of the liver-fed animals

It would appear from the data that the presence of basic nitrogen in a compound does not signify that its carcinogenic activity is limited to an organ or tissue remote from the site of application. It may act locally if given in sufficient concentration, over a long period of time, and in an oily medium.

Summary. 1. Sarcomata develop near the site of injection of *o*-aminoazotoluene given subcutaneously to mice. 2. Liver feeding does not appear to protect against the development of such sarcomata.

† We are indebted to Dr. E. E. Sproul of the Department of Pathology for interpretation of the histological sections.

[4] Personal communication from Roscoe B. Jackson Laboratory.

13551

A Tentative Test for Pyrogen in Infusion Fluids.

Co Tui and M. H. Schrift.*

From the Laboratory of Experimental Surgery, Department of Surgery, New York University College of Medicine.

Since Seibert[1] extended the work of Hort and Penfold[2] on the occurrence of pyrogen in distilled waters, various tests for the detection of this substance have been proposed. A chemical test which has been in use depends upon the decoloration of potassium permanganate added to the solution to be tested. But this test is unsatisfactory because, on the one hand, it can be used only in solutions in which medicaments do not decolorize this oxidizing agent and, on the other hand, it is not sensitive enough to detect the minute amount of pyrogen necessary to cause even a severe physiologic response. Resort must therefore be made to biologic test.

Test Animals. Seibert in her work used the rabbit as the test animal. This practice has been followed by other workers, among them Banks,[3] Lees and Levvy,[4] and by commercial houses in routine tests of infusion fluids. Sabin and Wallace[5] used the dog in their study of the chill-producing substance in pneumonia serum. While in all our published work[6] we have used the dog, we have had just as extensive experience with the rabbit. It is pertinent here to discuss the advantages and disadvantages of these 2 test animals.

The rabbit has a labile thermoregulatory mechanism and frequently gives false positive tests. Because of this, a negative test is more significant than a positive one in the rabbit. These animals must be conditioned to handling and to the taking of rectal temperatures for several days before they can be used, as untrained rabbits exhibit a rather bizarre temperature pattern. Feeding is followed by a mild pyrexia which lasts several hours and therefore they should not be fed for at least 6 hours before use as test animals.

* Aided by a grant from the New York Foundation.

1 Seibert, F. B., *Am. J. Physiol.*, 1923, **67**, 90; 1925, **71**, 621.

2 Hort, E. C., and Penfold, W. J., *Brit. M. J.*, 1911, **2**, 1589.

3 Banks, H. M., *Am. J. Clin. Path.*, 1934, **4**, 260.

4 Lees, J. C., and Levvy, G. A., *Brit. M. J.*, 1940, **1**, 430.

5 Sabin, A. B., and Wallace, G. B., Proc. Soc. Exp. Biol. and Med., 1929, **27**, 198; *J. Exp. Med.*, 1931, **53**, 339.

6 Co Tui, McCloskey, Schrift, and Yates, Proc. Soc. Exp. Biol. and Med., 1936, **35**, 297; *J. A. M. A.*, 1937, **109**, 250.

The dog has a much more stable thermoregulatory mechanism but is less sensitive to pyrogen than the rabbit. A positive test in the dog is so characteristic, however, with the additional symptoms of leucopenia, vomiting and diarrhea, that it is unmistakable. For this reason, a positive test in the dog is much more significant than a negative one.

To summarize, the rabbit is the better animal to test for the absence of pyrogen, while the dog is the better animal to establish the presence of pyrogen. Both animals must be used to complete the picture.

Sabin and Wallace[5] have shown that dogs react repeatedly to repeated intravenous injections of chill-producing pneumonia sera. In our experience both dogs and rabbits have been used for several positive pyrogen tests without developing manifest immunity or tolerance. Yet this question of immunity and tolerance has not been definitely settled, and until this is done it seems better to use a fresh animal for each test.

Another factor which has a direct bearing on the test is the sensitiveness to pyrogen of rabbits and dogs as compared with man. In preliminary studies with the purified pyrogen from typhoid vaccine, to be reported elsewhere, we found that the rabbit was approximately 3 times and the dog at least 6 times less sensitive than man.

Test Dosages. Assuming for the present that the above figures on comparative sensitiveness hold for pyrogen from other sources than the typhoid bacillus, the rabbit should receive as much as 3 times the maximum per kilo dose in man; and the dog 6 times.

Test doses of 5 to 20 cc per rabbit are employed by the commercial houses. On the assumption that the rabbits weigh 3 kg, this dosage would correspond to a 100 to 600 cc dose in man. If, in addition, comparative sensitiveness is taken into consideration, the dose is equivalent to 33⅓ to 200 cc. As a patient may receive as much as 2000 cc at a time, this test dose is manifestly inadequate. In order to approximate the dose in man, the rabbit test dose must therefore be not less than 50 to 100 cc per kilo body weight. This amount of fluid almost approximates the total blood volume of the animal, but if the dose is injected in 10 to 30 minutes, the rabbit can easily take care of the plethora. In any event, the symptoms of plethora are different from those of a pyrogenic reaction. As the dog is to be used as a positive test animal, any dose which elicits the reaction shows the degree of pyrogenicity of the solution. This can be arrived at by trial and error, starting from a minimum dose of 250 cc.

Procedure of the Test. The test is to be run in two parts, the preliminary part on rabbits, the confirmatory on dogs.

Preliminary Test. Two albino rabbits of 2½ to 3 kg are used. They must previously have been conditioned for several days to handling and taking of rectal temperature until these procedures provoke no struggling and their rectal temperature readings are constant within .25°C in 3 readings taken 15 minutes apart. Before injection, temperature readings taken at 15-minute intervals must not be over 39°C. In taking the temperatures, the thermometers must be inserted the same distance beyond the internal sphincter of the rectum and allowed to remain there for 2 minutes.

The test solution is warmed nearly to the temperature of the animals, and 50 cc per kilo body weight is injected into one animal and 100 cc per kilo body weight into the second animal, no faster than 10 cc per minute. The temperature readings are taken every half hour for 4 hours after the injection. A rise of over 5°C in 1 to 3 hours after injection is taken as presumptive evidence of the presence of pyrogen. A pyrogenic sample ought to give a more severe response with 100 cc than with 50 cc.

Confirmatory Test. This may not be necessary in situations, such as exist in commercial houses, where a positive presumptive test is enough to lead to the condemnation of a product as unfit for human use. In situations in which more positive evidence may be required, especially where there may be legal or medico-legal complications, the clear-cut result of a positive dog test is not easy to controvert. The test is performed as follows: One healthy dog of 15 kg body weight, conditioned to the taking of rectal temperature, with an initial rectal temperature of not over 39°C and blood count not exceeding 20,000 white blood cells per cu.mm, is used. Depending upon the response of the rabbits, from 250 cc to 1500 cc of the fluid is given intravenously through one of the external jugular veins, not faster than 30 cc per minute. The blood count is again taken 45 minutes and the temperature every half hour after the injection. The presence of a leucopenia (a fall of 5,000 cells per cu.mm or over) and temperature rise of .5°C from 1 to 3 hours after the injection are evidences of the presence of pyrogen. The occurrence of emesis, prostration and diarrhea are added checks.

Experimental Data. This two-fold test has been performed 214 times in this laboratory in a period of 32 months. Some of these tests were done for the New York University Surgical Division of the Bellevue Hospital, a few for some metropolitan hospitals, and the rest for the Comptroller's Office of the City of New York. Of

these tests, 172 were negative and the clinical use of these products has caused no pyrogenic reactions. Forty-two were positive, a result likewise confirmed by clinical use of the material.

The accompanying table embodies 5 sets of data obtained from the same commercial infusion fluid. Experiment 1 is the record of a pyrogenic reaction in a woman; Experiments 2 and 4 are the record of inadequate test doses in a rabbit and in a dog, respectively, while Experiments 3 and 5 are the record of adequate test doses in each of these 2 animals. The importance of using a test dose comparable to the human dose is shown by a study of Table I.

From the academic point of view it might be better to wait for more light on some of the factors previously mentioned before presenting the test. There is, however, a real need for a pyrogen test in the clinical world where inadequate tests are already in use. From this point of view, a test already given this amount of calibration and refinement would be better than the inadequately standardized tests now in use. Although this test may not always detect the presence of minute doses of pyrogen, it can be depended upon to detect the presence of moderately reactive amounts.

TABLE I.

Exp. No.	Test animal	Wt, kg	Dose per kg wt, cc	Total dose, cc	Change in WBC (in thousands)	Change in temperature, °C	Other symptoms
1.	F.C.C. ♀ Patient	55	11	600	8.-3.	37.7-41.8	Shivering, nausea, depression
2.	Rabbit	2.5	10	25		38.9-39.3	No symptoms
3.	Dog	12	20	240	21.5-14.4	38.7-39.1	No symptoms
4.	Rabbit	3	30	90		38.8-41.0	Depressed
5.	Dog	14	60	840	14.7-5.2	38.7-40.9	Emesis, diarrhea, depression

13552 P

Experimental Obesity in the Dog.

PETER HEINBECKER AND H. L. WHITE.*

From the Departments of Surgery and Physiology, Washington University School of Medicine, St. Louis, Mo.

During the past 5 years obesity in dogs frequently has been observed following operations on the hypophysis and hypothalamus. The operations, performed mostly through the oral approach, involved: (1) removal of the posterior lobe with or without removal of the pars distalis; (2) section of the fibers to the neurohypophysis with the immediate or subsequent removal of the adenohypophysis, the infundibular stem and the infundibular process; (3) removal of the neuro and adeno hypophysis at one sitting; (4) median transverse section of the posterior hypothalamus between the infundibular stem and mammillary bodies with or without removal of the posterior lobe and the pars distalis. The conclusions reported in this paper are based on observations made on 125 animals with a fairly equal distribution among the above classes.

The animals were kept in metabolism cages and 24-hour urine collections were made. They were fed measured limited amounts of dog chow and horse meat except when for experimental purposes unlimited but measured quantities were allowed. The dogs were kept for a period before operation to establish a constant weight and to determine the normal daily urine output. They then were subjected to operation and kept for periods of observation varying from 3 months to over a year. All but 6 of the animals have been sacrificed to obtain anatomical material to determine the lesions present in the hypophysis and in the hypothalamus. The brains were fixed *in situ* by irrigation through the carotid artery with formalin 1 to 10 after bleeding the animal. After further fixation the sella and its contents were separated from the hypothalamus. Both parts were then examined microscopically in serially cut 20-micron sections. Cresyl violet was used to stain the hypothalamic tissue, hematoxylin and eosin for the sella and its contents.

In this report are presented urine and weight charts (Fig. 1) and photomicrographs (Fig. 2) from 2 animals in each of which a lesion, caudal to the region of the paraventricular nuclei was made in the posterior hypothalamus. The pars distalis and posterior lobe

* Recipient of a grant-in-aid-of-research from the Commonwealth Fund.

also were removed in both, but the median eminence was not disturbed appreciably. These 2 dogs were chosen because of a critical difference in their lesions. In one, which became obese, the cells in the caudal portions of the paraventricular nuclei are absent (retrograde degeneration) in the animal which did not become obese the nucleus has its normal length. The shortening of the paraventricular nuclei in the obese dog is due to retrograde degeneration of the cells following the interruption of their axons by the posterior hypothalamic puncture. The puncture in the other dog did not sever these axons. The paraventricular nucleus gives rise to fibers going to the neural divisions of the hypophysis and to fibers which descend toward the brain stem.

Analysis of our entire material yielded evidence that obesity is dependent upon such a loss of cells in the caudal portion of the paraventricular nuclei. In addition a marked loss of cells in the supra optic nuclei appears to be a contributing factor in the development of maximal obesity. All the dogs in this series with marked diabetes insipidus became more obese than those partially diabetic but with a comparable degree of loss of cells from the caudal portion of the paraventricular nuclei alone. The degree of loss of cells of the supra optic nuclei is not critical in determining the degree of obesity. For instance, the degree of cell loss (87% estimated) in the supra optic nuclei of dog 0191 which did not become obese is somewhat greater than in dog 0190. The urine charts indicate that neither had significant diabetes insipidus but that the average percentage increase in urine output for dog 0191 was somewhat greater than for dog 0190. In 4 other dogs in which lesions were made in the hypothalamus between the stalk and the mammillary nuclei with a

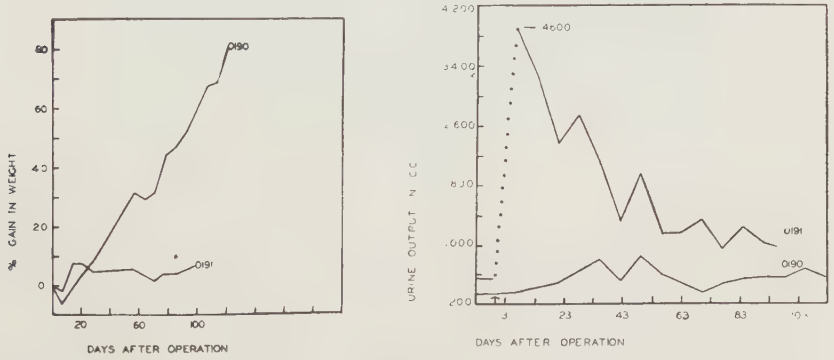


FIG. 1.

Plots of percentage increase in weight against time and of urine output in days after operation for dogs 0190 and 0191.

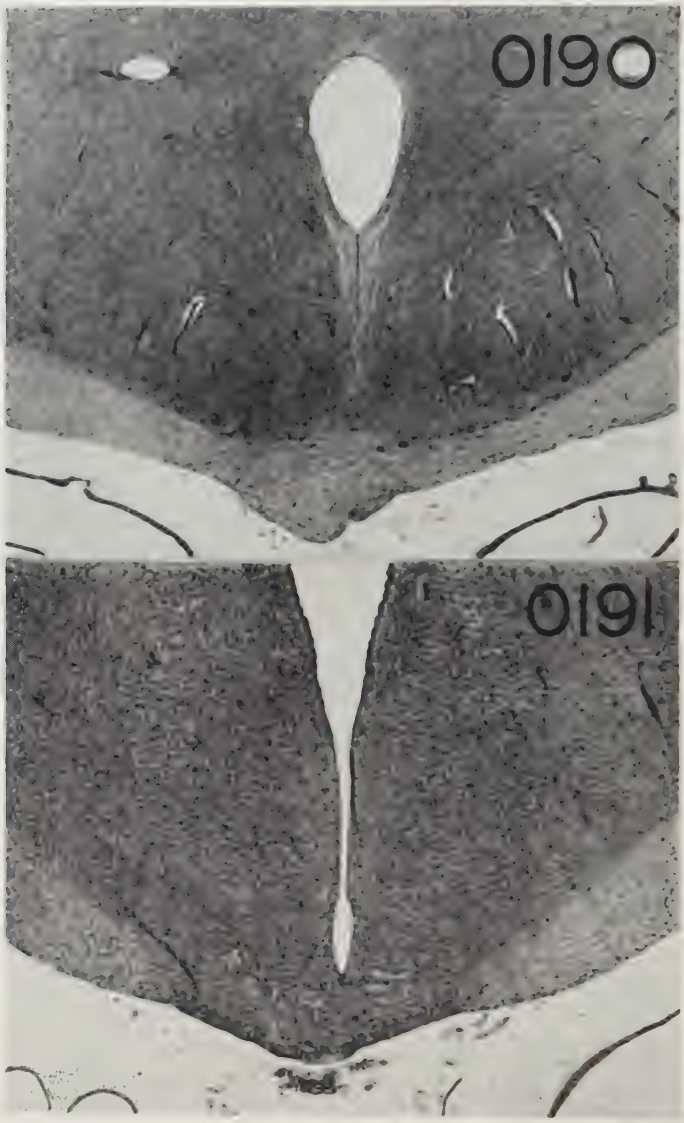


FIG. 2.

Photomicrographs, 12.5 diameters of frontal sections through the hypothalami of dogs 0190 and 0191. Note in dog 0190 the paraventricular nuclei have ended at a level still in the optic chiasma and more rostral than the section shown from dog 0191, caudal to the chiasma, in which the nuclei still are present.

resultant cell loss of considerable degree in the caudal portions of the paraventricular nuclei but with insufficient loss of cells from the supra optic nuclei to produce diabetes insipidus, the average weight

gain was 50% in 6 months. In other dogs with definite diabetes insipidus resulting from supra optic damage and with a comparable loss of cells in the caudal portions of the paraventricular nuclei the average weight gain has been 75 to 110%.

The conclusions drawn from an analysis of the entire material from which the above examples have been selected are: (1) Obesity in the dog results from partial destruction or retrograde degeneration of the paraventricular hypothalamic nuclei, particularly of their caudal portions. (2) Marked obesity results when destruction or denervation of the neurohypophysis and partial destruction of retrograde degeneration of the caudal paraventricular nuclei coexist. (3) Removal of the pars distalis in itself does not result in significant obesity (0 to 20%). The presence of the pars distalis in animals with partial destruction or degeneration of the supra optic and paraventricular nuclei is favorable to the development of marked adiposity. (4) The results suggest that a lack or marked lessening of the secretion of the neurohypophysis may aid in fat storage in the presence of a diminution in the number of cells of the caudal portion of the paraventricular nuclei.

13553 P

Body Fluid Changes in Neurogenic Hypertension and Total Paravertebral Sympathectomy.

Paul W. Schafer. (Introduced by D. B. Phemister.)

From the Department of Surgery, The University of Chicago.

This is a report of changes which have been observed in the blood volume, plasma volume, hematocrit and red blood cell count under various conditions of vascular tension resulting from operative interference with vasomotor nerve supply. These studies have been made on normal dogs, dogs made hypertensive by resection of both carotid sinuses, both aortic depressor nerve pathways, and one or the other vagus, and dogs made hypotensive by total paravertebral sympathectomy. Two hypertensive dogs have been subjected to total paravertebral sympathectomy and 2 sympathectomized dogs to the procedure used for production of hypertension and the subsequent changes noted.

Plasma volume was measured by the use of disappearance curves of the blue dye, T-1824. Blood volume was estimated from the plasma

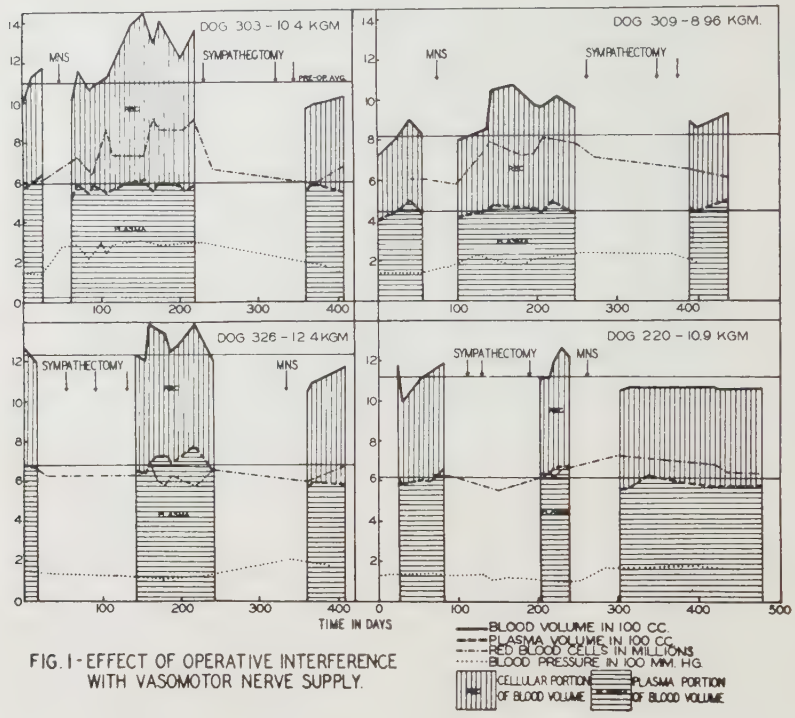


FIG. 1 - EFFECT OF OPERATIVE INTERFERENCE WITH VASOMOTOR NERVE SUPPLY.

volume and hematocrit. Blood loss encountered during each experiment was corrected by transfusion of an equal volume of blood from donor dogs.

As indicated in the accompanying graph, the following observations have been made:

First, that dogs subjected to modulator nerve section (MNS) develop marked hypertension and a markedly increased total blood volume apparently due entirely to an increase in the cellular fraction of the blood.

Second, that these hypertensive, polycythemic dogs regain approximately a normal total blood volume and their hypertension is markedly reduced after total paravertebral sympathectomy. This decrease in total blood volume after sympathectomy is apparently due to a decrease in cells alone, the plasma volume remaining unchanged.

Third, that dogs subjected to total paravertebral sympathectomy develop a slight hypotension and a slightly increased total blood volume due in the main to an increase in the plasma fraction of the blood.

Fourth, that these sympathectomized dogs, when subjected to

modulator nerve section develop a moderate hypertension not associated with any significant change in the total blood volume, either in its cellular or plasma fraction.

This work suggests a relationship between overactivity of the sympathetic nervous system and polycythemia. In those dogs with intact sympathetic efferent pathways, modulator nerve section was followed by a marked polycythemia and increased blood volume which disappeared after interruption of these sympathetic efferent pathways. Also in animals in which sympathetic efferent pathways had previously been interrupted, modulator nerve section caused no significant change in blood volume in either its cellular or plasma fraction.

At present the above described studies are being made on other dogs and, in addition, changes in their total body water and fluid available for the solution of sodium thiocyanate are being observed.

13554

Intravenous Nourishment with Protein Carbohydrate and Fat in Man.*

DWIGHT E. CLARK AND ALEXANDER BRUNSCHWIG.

From the Department of Surgery, The University of Chicago.

Intravenous injection of dextrose for both nutriment and hydration is now a common routine clinical procedure and the intravenous injection of casein digest to provide amino acids has also become feasible in man.[1] Intravenous injection of homogenized fat has been carried out, especially in infants, but is not commonly done at the present time.[2, 3]

In a patient having received an extensive resection of the larynx, upper trachea and most of the cervical esophagus for extensive carcinoma of the larynx, nutrition had been maintained by means of a Levine tube inserted into a sinus formed by the upper end of the esophagus in the base of the neck. With his coöperation this tube was withdrawn and for a period of 17 days all nutriment was given

* This work was conducted under a grant from Mead Johnson and Company, Evansville, Indiana.

1 Elman, E., and Weiner, D. O., *J. A. M. A.*, 1940, **112**, 796.

2 Holt, L. E., *J. Ped.*, 1935, **6**, 151.

3 Gordon, H., and Levin, S. Z., *Am. J. Dis. Child.*, 1935, **50**, 894.

intravenously with exception of the vitamins and liver extract. Four cubic centimeters of Vi Penta Oil (Hoffman-LaRoche) containing vitamins A, B_1, B_2, C and D were instilled every day into the open end of the esophagus. Liver extract was injected intramuscularly every third day. The parenteral solution for intravenous use consisted of "protein" in the form of an enzymatic hydrolysate of casein (Amigen) kindly furnished by Mead Johnson and Company, dextrose and highly emulsified fat. The patient was not bedfast but was up for several long intervals during the day sitting in a chair and walking about the ward.

The fat suspensions for intravenous use were prepared as follows: To 500 cc of the highest grade olive oil 250 g of freshly obtained commercial egg lecithin were added and the 2 mixed by stirring with a glass rod. After standing over night increased homogeneity occurred and the suspension was added to 4500 cc of triple distilled water. This mixture was then passed through a dairy homogenizer at 4500 pounds pressure (with kind coöperation of the Cherry-Burrell Corporation, Chicago). After this process the emulsion appeared as a "rich cream" slightly yellow in color. Microscopic study of the minute fat globules revealed that they were approximately 1 μ or less in diameter. The emulsion was then autoclaved for 15 minutes at 15 pounds pressure and stored in hermetically sealed flasks kept at room temperature. Several batches were prepared at succeeding intervals so that the emulsion used in this experiment was always less than 2 weeks old.

Two types of solutions were employed in this patient. (1) A mixture of 10% casein digest and 5 or 10% dextrose in distilled water or saline in variable proportions. (2) Fat emulsion added to the above with a maximum proportion as follows: 1000 cc of 10% dextrose in normal saline, 500 cc 10% solution of casein digest, 500 cc of the above described fat emulsion. Usually 2 injections were given daily, one in the morning and one in the evening at the rate of 500 cc an hour. Careful record was made of the intake and output in regard to nitrogen balance.

The accompanying table summarizes the intake and output during the 17-day experimental period and includes other pertinent data.

Plasma proteins, urine and stool nitrogen were determined in duplicate by the micro-Kjeldahl titration method. The values for non-protein nitrogen were always within the normal range.

While the quantities of carbohydrate, protein and fat administered were obviously inadequate to fully meet the standard requirements in this patient, he appeared surprisingly well during the experimental

TABLE I.
Patient A.Y. (No. 243693), ♂, 57 years.
Summary of Intake and Output During Period of Intravenous Nutrition.

Days	Weight, kg	Fluid intake, cc	Urine, cc	Dextrose, g	Fat, g	Protein, g	Nitrogen intake, g	Nitrogen excretion, g	Nitrogen balance, g	Calculated total calories	Salt intake,† g	Plasma proteins, g %
1	58.4											6.6*
2	59.7	2000	—	75	0	50	6	—	—	480	0	
3		3000	1050	175	0	100	12	7.1	+4.9	1060	0	
4	60.5	3000	975	175	0	100	12	7.7	+4.3	1060	0	
5		3100	1250	175	10	100	12	7.6	+4.4	1137	13.5	
6		3100	1050	200	70	100	12	11.5	+ .5	1700	3.6	
7	60.3	2000	—	75	0	50	6	—	—	480	0	
8		3000	1150	125	50	100	12	6.8	+5.2	1240	9.0	
9	61.2	3250	1590	140	0	100	12	8.8	+3.2	920	16.0	
10		2000	1100	100	50	50	6	7.6	—1.6	960	4.5	
11	59.9	1900	1100	100	0	50	6	4.9	+1.1	580	8.1	
12		2000	775	100	50	50	6	5.9	+0.1	960	4.5	6.4*
13	58.6	3300	—	160	0	80	9.6	—	—	930	8.1	
14	59.0	2000	—	100	50	50	6	—	—	960	4.5	
15		3000	1090	140	0	75	9	6.6	+2.4	830	9.0	
16	59.5	2000	742	100	50	50	6	3.0	+3.0	960	4.5	
17		3000	1065	140	0	75	9	5.8	+3.2	830	9.0	
18	58.4	2000	965	100	50	50	6	7.8	—1.8	960	4.5	6.5*

*R.B.C. and Hgb. comparable.
†Blood chlorides at beginning 98 Mm/L.; at end 107 Mm/L.

period. At no time did he complain of hunger but especially on the seventh and eleventh day, stated that he "felt dry." The fact that there was a positive nitrogen balance may be explained on the basis of avidity of the organism for nitrogen. As a result of his disease causing reduction in adequate intake over a prolonged period, there was probably depletion of body nitrogen stores.

As shown, fat was injected on 8 different occasions and the urine excreted during each of the subsequent 24 hours contained no fat droplets and total lipid determinations on a large aliquot of the pooled urine revealed no fat. There were no symptoms attributable to fat embolism. At no time was there abdominal or chest pain or rise in temperature. A moderately severe reaction developed in the arm with thrombosis in several veins. This reaction occurred only near the end of the experimental period and cleared up promptly when the injections were stopped.

One year after this experiment the patient, having nourished himself during this survival period by esophageal tube, died of hemorrhage from the left carotid artery which had been invaded by recurrent carcinoma. Necropsy revealed no visceral abnormalities except

for areas of *old* calcification in the pancreas unaccompanied by acute inflammation. In view of the absence of abdominal pain during the period of intravenous injection and during the subsequent 12-month survival there is no basis for assumption that such changes were due to pancreatitis incident to these injections.

That the injected nutriment was actually utilized is strongly suggested by (1) the positive nitrogen balance and (2) the maintenance in weight. The latter could not be accounted for on the basis of hydration because the patient frequently experienced thirst and the daily urine output was 1000 cc or more on a fluid intake of 2000 to 3000 cc.

Summary. Parenteral nutrition was carried out over a 17-day period in an adult male by means of intravenous injection of dextrose, casein digest (amino acids) and emulsified fat. It is believed that this is the first recorded instance of nutrition in man by the simultaneous administration of the 3 primary types of foodstuff.

13555 P

Potassium Chloride Therapy and Serum Potassium in Infantile Eczema.*

A. V. STOESSER.

From the Department of Pediatrics, University of Minnesota.

The observation has been made that the administration of potassium chloride to young children with bronchial asthma has given relief in some instances, especially when the intake of sodium chloride has been held at a low level. The ingestion of the potassium salt causes a cessation of the milder attacks of asthma, but in the more severe cases the asthma continues while the potassium chloride is being taken but ceases almost at once when the salt is discontinued.[1] Some of these subjects have had eczema during infancy and up to the time of the onset of the asthma. They appeared to respond best to the potassium chloride therapy.

Recent writings on eczema have emphasized the nonspecific factors as a cause of the disease. The skin of the infant especially the horny

* This work was supported by a grant from the Medical Research Fund of the University of Minnesota.

1 Stoesser, A. V., and Cook, M. M., *Am. J. Dis. Child.*, 1940, **60**, 1252.

layer is thinner than that of the older child and the terminal capillaries show differences of configuration. The infant's skin has a higher content of water and of sodium chloride and a lower potassium/sodium ratio.[2] These features tend to disappear with growth but in the child with eczema they may remain or change slowly. The administration of potassium to increase the potassium/sodium ratio has been recommended. No careful studies have been made to determine the value of this nonspecific form of therapy in infantile eczema.

Six infants with eczema ranging in age from 7 to 16 months were chosen and placed under constant environmental conditions in the hospital. Nurses carefully instructed in the care of these little patients, bathed them with mineral oil, and applied only *unquentum acidi borici*. To prevent scratching it was necessary to use sedatives such as chloral hydrate. The infants were given an evaporated milk formula consisting of equal parts of irradiated unsweetened evaporated milk and distilled water. The younger children had 7% dextrimaltose added to the formula. The older subjects were offered a variety of the precooked cereals together with plenty of vegetables and fruits. Eggs were not permitted in any of the diets.

The type of eczema was practically the same in each case. There was a moist, red surface exuding serum. The disease was chiefly confined to the face and upper trunk, and to a lesser extent to the extremities. Three of the patients received potassium chloride orally, starting with 5 grains 3 times a day and increasing gradually over a period of one week to 15 grains 4 times a day. Then the salt was discontinued. The remaining 3 cases were observed as controls.

Samples of blood for the determination of the serum potassium, sodium and chloride were obtained shortly after admission during the acute stages of the disease, 2 weeks later when the condition of the skin had improved and again 4 weeks later when weeping had ceased entirely. The chemical methods used for the analyses of the blood were as follows: Potassium, Shohl and Bennett[3]; sodium, Butler and Tuthill[4]; and chloride, Wilson and Ball.[5]

The results are summarized in Table I.

The ingestion of potassium chloride does not improve the eczema, but instead causes a slight aggravation of the condition of the skin. The serum potassium is above the normal range during the more

[2] Holt, L. Emmett, Jr., and McIntosh, Rustin, *Holt's Diseases of Infancy and Childhood*, New York, D. Appleton-Century Company, 1940, Edition 11, 899.

[3] Shohl, A. T., and Bennett, H. B., *J. Biol. Chem.*, 1928, **78**, 643.

[4] Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, **93**, 171.

[5] Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, 1928, **79**, 221.

TABLE I.
Serum Electrolytes During Acute Phase and Various Stages of Healing in Infantile Eczema.

Case No.	Shortly after admission			End of 2nd hospital wk			End of 1st hospital mo.		
	K	Na	Cl	K	Na	Cl	K	Na	Cl
	Mg per 100 cc serum								
Cases receiving potassium chloride orally									
1	25	289	366	24	320	378	16	298	354
2	25	336	369	27	318	365	20	297	319
3	24	314	366	21	303	344	14	305	360
Avg	24	313	367	24	313	362	16	300	344
Controls receiving no potassium chloride									
4	27	335	399	24	302	348	19	298	366
5	23	312	360	20	307	381	15	309	375
6	29	318	377	24	313	395	15	323	374
Avg	26	321	378	23	307	374	16	310	371

severe phases of the disease. This elevation is present in infants receiving the potassium chloride as well as in the controls. With improvement in the eczema, the potassium of the blood begins to fall and when all oozing has disappeared leaving the skin dry, the values are normal. The serum sodium and chloride are normal during the acute stages of the eczema, and do not undergo any significant change as healing takes place. The administration of potassium chloride causes no change in the sodium and chloride of the blood. That the levels of these electrolytes are normal was established by examining 24 samples of blood from 12 children ranging in age from 4 months

TABLE II.
Serum Electrolytes in a Group of Infants and Young Children Who Have Never Had Eczema.

Case No.	Admission to hospital			Shortly before discharge		
	K	Na	Cl	K	Na	Cl
	Mg per 100 cc serum					
1	18	323	380	14	317	362
2	17	313	357	18	—	368
3	16	317	371	17	312	352
4	18	315	365	18	313	348
5	16	320	370	17	320	357
6	16	315	381	16	315	376
7	17	318	356	15	319	351
8	18	300	341	19	298	364
9	17	332	382	18	342	387
10	16	340	392	16	326	398
11	16	333	400	15	315	351
12	11	322	369	17	323	380
Avg	16	320	372	16	318	366

to 2 years. None of these subjects had any skin or allergic disease. The results are shown in Table II.

The elevation of the serum potassium when the eczema has progressed to the point where the papules of the disease coalesce and produce a moist red surface exuding fluid is a most interesting observation. These little patients certainly must have at this stage a marked disarrangement of the normal electrolyte balance. The threshold for the excretion of potassium is raised subjecting the tissue cells including those of the skin to a high potassium content. Water must be drawn into the cells. Swelling takes place with some cell disruption. This may cause the extreme irritability of the skin which is present at the height of the disease. It is in line with the studies of Klauder and Brown,[6] who noted that in an examination of the content of calcium, potassium, magnesium and sodium in rabbit skin in relation to cutaneous irritability as judged by the applications of croton oil, there was an inverse relation between irritability of the skin and calcium content and a direct relation in regard to potassium. The greater the irritability of the skin the greater the likelihood of a low calcium and a high potassium content. Potassium chloride therapy, therefore, does not appear to be indicated in infantile eczema.

13556

Infection of Horses with St. Louis Encephalitis Virus, Experimental and Natural.*

W. McD. Hammon, B. N. Carle and E. M. Izumi. (Introduced by K. F. Meyer.)

From the George Williams Hooper Foundation and Division of Veterinary Science, University of California, San Francisco and Berkeley, California.

Within the last year evidence has been obtained indicating a close epidemiological parallel between the viruses of the "equine" encephalitides and that of St. Louis encephalitis. Howitt[1] first called attention to antibodies to both Western equine and St. Louis viruses in the blood of certain patients in California. More recently, Ham-

6 Klauder, J. V., and Brown, H., *Arch. Dermat. and Syph.*, 1927, **15**, 1.

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Howitt, B. F., *Am. J. Pub. Health*, 1939, **29**, 1083.

mon,[2] and Hammon and Howitt[3] pointed out that human infections apparently due to both viruses were occurring during the same epidemic, and possibly even simultaneously in the same individual. This occurred during an epidemic in the Yakima Valley, Washington. Howitt,[4] over a period of several years, collected a large number of horse sera from California, a high proportion of which neutralized both the Western equine and the St. Louis viruses. Philip, Cox and Mountain[5] noted St. Louis virus antibodies in horses in Colorado, Montana and Washington. The same year Hammon and Howitt[3] made a similar observation in Washington.

In the Yakima Valley, Hammon, Reeves, Brookman, Izumi and Gjullin[6, 7] isolated repeatedly both the St. Louis and the Western equine viruses from the same species of mosquito, *Culex tarsalis,* and from no other species, though all biting arthropods found were tested. Further evidence of this close epidemiological relationship was obtained in an extensive survey of mammals and birds in this area by Hammon, Gray, Evans, Izumi and Lundy.[8, 9] Among approximately 600 sera tested, from domestic and wild species, equal proportions were found positive to the St. Louis and to the Western equine viruses. As was the case with the equine virus, a much higher percentage of horses and mules had antibody to the St. Louis virus than was found in samples of any other species of mammals or birds. It was not surprising, therefore, to learn of experimental confirmation of the susceptibility of horses to the St. Louis virus.

Cox, Philip and Kilpatrick,[10] using a St. Louis strain isolated by Webster, inoculated horses by the intracerebral route. Those without previous serum antibody for this virus developed clinical encephalomyelitis, fatal in some instances. During the terminal phase

[2] Hammon, W. McD., *J. A. M. A.*, 1941, **117**, 161.

[3] Hammon, W. McD., and Howitt, B. F., *Am. J. Hyg.*, 1942, **35**, 163.

[4] Howitt, B. F., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 247.

[5] Philip, C. B., Cox, H. R., and Mountain, J. H., *Pub. Health Rep.*, 1941, **56**, 1388.

[6] Hammon, W. McD., Reeves, W. C., Brookman, B., Izumi, E. M., and Gjullin, C. M., *Science*, 1941, **94**, 328.

[7] Hammon, W. McD., Reeves, W. C., Brookman, B., and Izumi, E. M., to be published.

[8] Hammon, W. McD., Gray, J. A., Jr., Evans, F. C., Izumi, E. M., and Lundy, H. W., *Science*, 1941, **94**, 305.

[9] Hammon, W. McD., *Proc. Calif. Mosq. Abatement Soc.*, 1941.

[10] Cox, H. R., Philip, C. B., and Kilpatrick, J. W., *Pub. Health Rep.*, 1941, **56**, 1391.

of the disease they succeeded in isolating the virus from nasal washings of one animal. However, although bleedings were made at frequent and regular intervals, at no time did they isolate virus from the blood of any animal.

It should be pointed out that in these experiments an artificial route of infection was employed, and that the virus used had been thoroughly adapted to brain tissue through repeated serial intracerebral passage in mice. As yet, in no instance has this virus been isolated from the brain of a horse suffering from a naturally acquired encephalitis. Furthermore, large numbers of horses which two of us (W. M. H. and E. M .I., Table I) have tested and also those which Howitt[4] has tested and found to have antibodies have for the most part been horses which have never had recognized encephalitis.

During the fall of 1940 we began a search for a satisfactory group of horses to inoculate. Not till late in the summer of 1941 was a group found available for purchase that did not already possess serological evidence of immunity. In Table I is presented the list of horses tested during the fall of 1940 and summer of 1941 with the results of the tests. This list includes all sera tested, some collected or submitted for various purposes. The sera from Massachusetts furnished the first evidence that neutralization was not a normal characteristic of horse serum. The 4 wild, unbroken colts from an uninhabited mountainous Nevada area were imported for the experiments which are here described.

Method. A St. Louis virus (Culex 103) isolated from *Culex tarsalis*[6] was used in all experiments. It was employed in its first and second mouse brain passage, and suspended in broth containing

TABLE I.
Horse Bloods Tested for Antibody to St. Louis Encephalitis Virus.

Source	Negative	Positive
[1]Berkeley, Calif., Agr. Exp. Sta.	0	1
[2]Davis, Calif., Agri. Exp. Sta.	0	12
[3]Boston, Mass., Biol. Laboratory, State Health Dept.	4	2
[4]Los Angeles, Calif., Stock Yards	1	1
[5]Nevada, Various Ranches	0	3
[5]Reno, Nevada, Agri. Exp. Sta.	3	3
Texas, Southeastern Part	4	4
Pinal County, Arizona	1	0
Yakima, Wash.	3	23
[5]Nevada, Mountainous Area—Wild Colts	4	0
Total	20	49

Note: We wish to acknowledge the kind coöperation of the following persons in collecting and shipping certain of these sera: [1]Dr. C. M. Haring, [2]Dr. Wilson B. Bell, [3]Dr. E. S. Robinson, [4]Dr. L. M. Hurt, and [5]Dr. L. R. Vawter.

5% sheep serum. In the horses, intranasal and intracerebral injections were made under general chloral hydrate anesthesia, given intravenously. Temperatures were taken twice daily. Bleedings were made from the jugular vein, and the blood defibrinated and stored at 5°C until inoculated. Nasal washings were obtained by carefully and thoroughly swabbing each nostril with a cotton ball soaked in saline. The saline washings were centrifuged at 16,000 to 18,000 rpm for 10 minutes before inoculation. All inoculations were made intracerebrally in groups of 5 Swiss mice. In the case of those showing encephalitic symptoms of dying, the brains were removed and if sterile by bacteriological tests, were ground up and passed to a second group of mice. If these all succumbed with typical signs and with the customary incubation period, the identification was considered sufficiently well established. Neutralization tests on sera from recovered horses were made by mouse inoculation according to the method described by Hammon and Izumi.[11]

Experiment 1. A 1-year-old filly was given 3.0 cc of a 10% virus suspension intranasally. Another 1-year-old filly was inoculated subcutaneously (very superficially) with 3.0 cc of a 10^{-4} dilution of the same virus. This represented about 100 to 1,000 fatal intracerebral mouse doses—planned to approximate a possible single mosquito inoculation. Two uninoculated control animals were kept in a separate corral. All were bled for serum neutralization tests just before inoculation and again 47 days later. Temperature taking, bleeding and nasal washing were begun 3 days after inoculation and continued for 21 days. In the inoculated animals, no signs of illness were noted, no elevation of temperature occurred, and no virus was isolated from blood or nasal washings. The preinoculation sera were found free from detectable antibody. The inoculated horses developed a high titer of antibody, but the controls did not.

Experiment 2. Fifty-seven days after the inoculation of the 2 fillies, these and one of the previous controls—a 2-year-old stallion—were each inoculated intracerebrally with 1.0 cc of a 5% suspension of virus. The other previous control—also a 2-year-old stallion—was inoculated subcutaneously (very superficially) with 5.0 cc of a 1% virus suspension. Bleeding was begun 12 hours after inoculation, and each was bled twice daily thereafter for 15 days. Nasal washings were first taken 24 hours after inoculation and made once daily thereafter. No elevations of temperature occurred in any animal except a slight rise during the 2 postoperative days in those

[11] Hammon, W. McD., and Izumi, E. M., *J. Immunol.*, 1942, **43**, 149.

inoculated intracerebrally, and no signs of illness were noted, although these observations were continued for 22 days. No virus was isolated from the nasal washings. However, from the stallion receiving the subcutaneous inoculation, the virus of St. Louis encephalitis was isolated from the blood 26, 36, and 48 hours after inoculation, and from the stallion inoculated intracerebrally virus was isolated 26 hours after inoculation. The blood of both of these horses, drawn just prior to inoculation, failed to neutralize but 24 days later both had developed a high titer of antibody.

Discussion. In the first experiment we obviously erred in waiting till the third day to begin bleeding. In the second experiment it was demonstrated that virus circulates in the blood between 24 and 48 hours after inoculation. It is highly improbable that this represents only a survival of the inoculated virus, for *in vitro,* between the factors of enormous dilution and storage at above 37°C, no detectable amount of virus could be expected to survive. It appears more probable that the virus underwent multiplication in some tissue. The prompt development of antibody from inoculation of a single dose of virus in all inoculated animals is further evidence of infection.

In every instance, whether by subcutaneous, intranasal or intracerebral inoculation, no clinical disease was noted. This, with the finding of circulating virus, is in marked contrast to the findings of Cox, Philip and Kilpatrick, and probably was due to the difference in the tissue adaptation of the two viruses. Our virus, from all evidence at hand, produces in nature through the mosquito vector, just such an inapparent infection in many different animals, including the horse, as we have demonstrated in the horse. This infection is associated with circulating virus to permit infection of other mosquitoes. In this cycle there is no evidence that the virus multiplies in or invades neural tissue. The virus employed by Cox and his associates was definitely adapted to growth in neural tissue, and when inoculated directly into this tissue in horses resulted in encephalomyelitis.

We might reasonably conclude then, from all the data at hand, that the St. Louis virus is as much an "equine" virus as those called Eastern and Western strains, but manifests neurotropic tendencies in the horse much less frequently than these others.

Finding virus in the blood greatly strengthens the case for mosquito transmission of the St. Louis virus.

Summary. Horses from many of the Western States and from Massachusetts have antibodies, in varying proportion, to the St. Louis encephalitis virus. Inoculation in non-immune horses by the

subcutaneous, intranasal or intracerebral routes, of a St. Louis virus freshly isolated from mosquitoes, failed to produce clinical disease. It did produce inapparent infection resulting in the presence of virus in the blood, in 2 instances in which suitable tests were made, and in all 4 inoculated animals induced a high titer of serum antibody. We conclude that in horses, inapparent infection occurs frequently in many widely scattered areas of the United States.

Acknowledgment. Valuable technical assistance was rendered by Mr. V. W. White.

13557 P

Effect of Estradiol-Benzoate on Serum Lipid of Rats Consuming a High Fat Diet.

HAROLD G. LOEB.* (Introduced by G. O. Burr.)

From the Departments of Physiological Chemistry and Botany, University of Minnesota, Minneapolis.

The ability of estrin to induce a lipemia in the male fowl was demonstrated by Lorenz, Chaikoff, and Entenman.[1] This was confirmed by Zondek and Marx,[2] who not only showed a marked lipemia but also the accumulation of fat in the internal organs, particularly the liver. Very large doses were employed of the order of 40,000 I.U. (4 mg estradiol-benzoate) given on 2 successive days. Their results with mammals were negative. They could not induce lipemia in rabbits, rats, and man with large doses of estrin. The work reported below is part of a larger experiment and is presented, primarily, to show that an increase of serum lipid in rats is possible with estradiol-benzoate.

Methods. Rats were taken at weaning time and placed on low fat diets for 4 weeks. During the subsequent 8 weeks which comprised the experimental period the special diets and estrogenic hormone were administered. One group received the fat-free, high carbohydrate diet 550-B;[3] a second group received the balanced ration containing 20% lard, diet 560-B;[3] and the third group was

* Rockefeller Foundation Fellow.

[1] Lorenz, F. W., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, 1938, **126**, 763.

[2] Zondek, B., and Marx, L., *Arch. Int. Pharmacodyn.*, 1939, **61**, 77.

[3] Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, **82**, 345.

given a high fat diet, 580-B,[4] with the fat (71%) supplied as hydrogenated coconut oil (no unsaturated fatty acids were included in the diet). The same supplements were provided with all 3 diets. Estradiol-benzoate† was administered only to male rats receiving diet 580-B, as follows: In subgroup 1 each rat received subcutaneously 300 μg administered in 100 μg doses‡ (1000 I.U.§) on alternate days during the last week of the experimental period. Those in subgroups 2 and 3 were given 30 μg (300 I.U.) and 5 μg (50 I.U.), respectively, every day except Sunday during the last 4 weeks of the experimental period (24 injections for each animal in each subgroup). All of the rats were anesthetized with ether 24 hours after the last injection and blood was taken from the abdominal aorta. The blood obtained from each group of 3 animals was pooled. After centrifugation the serum was removed and analyzed for total lipid‖ by means of the oxidative method described by Boyd.[5]

Results. Animals receiving the high fat diet showed a higher average value for serum lipid than those on the balanced and fat-free rations. With rats in subgroup 1, which received large amounts of the hormone over a relatively short period, the value for serum lipid did not exceed that observed in the controls. This is contrary to Zondek's observation on cocks in which equivalent amounts of hormone over a short interval caused a large increase in the blood lipid value. However, subgroups 2 and 3 which received smaller doses over a longer interval showed a definite, though moderate, increase in serum lipid. The negative result in subgroup 1 indicates that rats are much more resistant to the estrogen effect than fowl. Animals in subgroup 3, although they received in all less hormone (120 μg) than those in subgroup 1 (300 μg) exhibited the lipemic response.

Whether the positive estrogen effect is due to the nature of the diet or the deficiency of essential fatty acids, or possibly both, can not be ascertained from the data at hand. Zondek and Marx[2] report that in cocks, at any rate, the effect of estrogenic hormone on the blood lipid level is not significantly influenced by the composition of the diet.

---

[4] Loeb, H. G., Ph.D. Thesis, University of Minnesota Library, 1940.

† The authors wish to thank Dr. R. J. Floody (Roche-Organon, Inc.), who kindly supplied the estradiol-benzoate.

‡ Each dose in every case was contained in 0.05 cc of olive oil.

§ International unit for benzoylated forms of pure estrogens = 0.1 μg of α-estradiol-3-monobenzoate. *Quart. Bull. Health Organization of League of Nations*, 1935, **4**, 618.

‖ *i. e.*, total fatty acids plus total cholesterol.

[5] Boyd, E. M., *Am. J. Clin. Path.*, 1938, **8**, 77.

TABLE I.
Serum Lipid in Rats Receiving Estradiol-benzoate (mg%)

Diet	Group	Total estradiol-benzoate (μg)	Total* lipid (mg%)	Avg
580-B	Controls ♂	—	363 403	351
	" ♀	—	386 251	
580-B	Sub-group 1 ♂	300 (3)†	366 301	334
	" " 2 ♂	720 (24)	578 553	566
	" " 3 ♂	120 (24)	521 541	531
560-B	♂	—	251	252
	♀	—	252	
550-B	♂	—	213	246
	♀	—	278	

*Each figure was obtained on the serum from 3 pooled samples of blood. Figures refer to total fatty acids plus total cholesterol.

†Figures in parentheses represent the number of injections.

Summary. A moderate lipemia was induced in male rats with subcutaneous injections of estradiol-benzoate. The animals were maintained on a diet rich in fat (71% hydrogenated coconut oil) but deficient in essential fatty acids.

13558

Physiological Properties of the Reynals Testicular Diffusion Factor.

FRANCIS X. AYLWARD.* (Introduced by L. Emmett Holt, Jr.)

From the Department of Pediatrics, Johns Hopkins University, School of Medicine, Baltimore, Md.

We have previously described[1] some chemical properties of the diffusion factor from mammalian testicle. This substance, discovered by Duran Reynals,[2] can be demonstrated by injecting a

*Commonwealth Fund Fellow in Biochemistry.

1 Aylward, PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 477.

2 Duran Reynals, *Compt. rend. Soc. Biol.*, 1928, **99**, 6.

small amount (*e. g.*, 0.2 ml) of an aqueous solution intradermally into the shaven skin of a rabbit; the extract spreads *immediately* over a large area, whereas a corresponding injection of saline or Ringer's solution remains localized as a bleb in the dermis for some time. The rate of disappearance of the bleb following the injection of an extract can be used as a rough test of activity. When an active extract mixed with an "indicator" (*e. g.*, India ink, hemoglobin, various dyes or diphtheria toxin) is injected intradermally, the indicator spreads over a large area; from such experiments methods of assay have been developed notably by Madinaveitia[3] and Bacharach[4] using hemoglobin. The present experiments were designed to investigate some physiological properties of the diffusion factor. It was also hoped to develop a more sensitive method of bioassay than was yet available.

Extracts were prepared as previously described[1] from the powder 4H, an acetone precipitate of an aqueous extract of beef testicles.

1. *Increase in Capillary Permeability after Intradermal Injection.* Five rabbits were given an intravenous injection of 5 ml of a 1% solution of the blue dye T. 1824. Each rabbit was then given 2 intradermal injections, one of 0.5 ml saline and a second of 0.5 ml of 5% saline solution of testicular extract (T.E.). Colored patches appeared in the skin round the site of the T.E. injections, showing that the dye was able to pass through the capillaries in the presence of the extracts, whereas at the control sites only a very slight coloration was seen. Similar results were obtained with intradermal injections in adult dogs and puppies.

The results, in agreement with those of Duran Reynals,[5] indicated that T.E. exercised a local effect on capillary permeability, but this cannot be regarded as a specific test for the diffusion factor as other substances producing local capillary damage have a similar effect.

2. *Increase in Capillary Permeability after Intravenous Injection.* Five ml of a 1% solution of T. 1824 were injected into the ear veins of each of 2 rabbits, followed by intravenous injections of 10 ml of 10% T.E. in one animal and 10 ml saline in the other. The experiments were repeated with further pairs of rabbits using 15 ml and 20 ml of T.E. The rabbits were killed 30 minutes after the injections.

The skins of all the rabbits were colored slightly at the end of 30 minutes, the coloration being particularly obvious in the ears and

[3] Madinaveitia, *Biochem. J.*, 1938, **32**, 1806.

[4] Bacharach, Chance and Middleton, *Biochem. J.*, 1940, **34**, 1464.

[5] Duran Reynals, *Yale J. Biol. and Med.*, 1939, **11**, 601.

nostrils, but the rabbits given T.E. developed skin coloration much more quickly and the final color was more intense. Similar observations were made on groups of mice given injections of 0.2 ml T.E. and 0.2 ml T. 1824 through the tail vein.

In agreement with the results of Duran Reynals[5] changes were also observed in the tissues of both rabbits and mice, the group given T.E. showing a pronounced degree of coloration in liver and lungs.

Experiments were then carried out in collaboration with Dr. M. I. Gregersen to see if this effect could be measured quantitatively, using a modification of the blood volume method of Gregersen.[6] In adult dogs (body weights about 2 kg) 2 ml of a 1% solution of the dye T. 1824 were injected into the jugular veins and blood samples were taken at intervals of 10 minutes over an hour. The dye content of the blood was measured, using a spectrophotometer and the results plotted graphically, the normal rate of loss of T. 1824 from the circulation being calculated for each animal. One hour after the dye injection, a sterile 5% solution of T.E. in amounts up to 200 ml was injected into the jugular vein over a 5-minute period. There were no obvious toxic symptoms. Blood samples were then taken at intervals over an hour and the dye concentrations plotted against time.

It was hoped that the effect of T.E. on permeability would be demonstrated by an increase in the gradient of curves, but apart from slight irregularities immediately following the injections there were no significant changes.

At the end of both experiments 0.25 ml injections of 5% T.E. were given intradermally to each dog, and within 5 minutes a blue ring appeared round the site of each injection, the central area being slightly red. The blue rings increased in size; the central area became smaller and in about 15 minutes had disappeared, so that a blue circular patch about 3.5 cm diameter resulted. The colored area continued to increase for some hours.

The experiments were repeated on puppies, no significant changes in the blood dye curve being found. 0.25 ml T.E. given intradermally to the puppies produced results similar to those in the adult dogs except that the skin colorations were even more marked.

The negative results following intravenous injection of the extracts may be due to partial inactivation of the beef testicular extracts in the blood stream of the dogs; they are more probably to be explained in terms of dosage. It was evident that no sensitive biological assay for T.E. could be developed on these lines.

[6] Gregersen, *J. Lab. and Clin. Med.*, 1938, **23**, 423.

3. *Experiments with the Clark Rabbit Ear Chambers.* In collaboration with Dr. R. G. Abell[7] experiments were performed on rabbits in the ears of which a moat chamber had been fitted, permitting microscopic observations of the effect on the capillaries of extracts placed in the moat. When testicular extracts (0.15 ml of 5% sterile solution of 4H) were placed in the moat, a striking increase took place in the permeability of the capillaries in the ear chamber, to such an extent that fluid passed through the vessel wall, leaving the corpuscles and part of the plasma concentrated within the capillaries. The concentration was sufficiently great to stop the flow of blood through some of the capillaries, but not sufficient to cause loss of outline of the corpuscles. The main effects were observed within 10 minutes and did not increase with continued contact of the extracts with the vessels.

In further experiments 142 mg/kg of T. 1824 was injected intravenously into each rabbit, and the rate of passage from the capillaries into the surrounding tissue was observed. Control studies by Abell[8] have shown that the dye cannot normally be seen outside the blood vessels until approximately 100 minutes after intravenous injection. When, however, 0.15 ml of sterile 5% T.E. were added to the moat its effect was readily seen under the microscope and within 13 minutes sufficient of the dye had passed out of the vessels to be readily visible in the tissues.

These experiments confirm in a very direct way the effects of testicular extracts on capillary permeability.

4. A number of other properties of testicular extracts have been studied. The ability of T.E. to increase absorption through the skin was tested by means of an insulin ointment in which T.E. was incorporated. Negative results were obtained. T.E. was found to be without effect on the contraction of the isolated guinea pig uterus and rabbit intestine. Injections of T.E. preparations produced no conspicuous changes in the blood pressure of experimental animals (dogs, cats, rabbits).

Since the above experiments were carried out, the observations of Chain and Duthrie[9] on the mucolytic activity of testicular extracts has led to the conception that the diffusion factor is identical or at least closely allied to hyaluronidase and that the diffusion is due to the action of this enzyme on the hyaluronic acid (a muco-polysaccharide)

7 Abell and Aylward, *Anat. Rec.*, 1941, **79** (Suppl. 2), 1.

8 Abell, R. G., *Anat. Rec.*, 1940, **78**, 214.

9 Chain and Duthrie, *Brit. J. Exp. Path.*, 1940, **21**, 324.

present in normal skin and other tissues.[10] This recent work has been reviewed[10, 11] and will not be discussed here.

Conclusions. The experiments described indicate that testicular extracts have the property of increasing capillary permeability. No evidence was found that they could increase absorption from the skin, nor were pharmacological effects on smooth muscle or blood pressure demonstrable.

13559

Observations on Resistance of *Staphylococcus aureus* to Action of Tyrothricin.

CHARLES H. RAMMELKAMP. (Introduced by Chester S. Keefer.)

From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston, Mass.

Tyrothricin, the crude substance isolated by Dubos from a spore-bearing soil bacillus has been found to kill gram-positive micro-organisms both *in vitro* and in the experimental animal.[1, 2] It is now known that tyrothricin contains 2 crystalline fractions, tyrocidine and gramicidin, the latter exhibiting the greater bactericidal action *in vitro* and *in vivo*.[3] Because of the difficulties encountered in preparing crystalline gramicidin, tyrothricin has been used almost exclusively in the treatment of infections.

Although tyrothricin is active against all gram-positive organisms, the different genera vary considerably in their susceptibility to its action. It is now well established that pneumococci and streptococci are most susceptible *in vitro* whereas staphylococci require somewhat greater concentrations of tyrothricin before a definite killing effect is observed.

During the past 2 years we have used tyrothricin in the treatment of experimental and clinical infections[2, 4] caused by the pneumococcus, streptococcus, and staphylococcus. Early in these studies the observation was made that it was difficult to predict from the *in*

10 Meyer and Palmer, *J. Biol. Chem.*, 1936, **14**, 689.

11 Madinaveitia and Quibell, *Biochem. J.*, 1940, **34**, 625.

12 McClean and Hale, *Biochem. J.*, 1941, **35**, 159.

1 Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 11.

2 Rammelkamp, C. H., to be published.

3 Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, 1941, **73**, 629.

4 Rammelkamp, C. H., and Keefer, C. S. (abstract), *J. Clin. Invest.*, 1941, **20**, 433.

vitro susceptibility of staphylococci in general the amount of tyrothricin required for the sterilization of a local infection caused by this organism. There appeared to be 2 possible explanations for this difficulty; either (1) the strains were resistant to tyrothricin or (2) certain inhibitory substances were present in the infected area which prevented the action of tyrothricin on staphylococci. Both of these factors were found to play an important rôle in determining the effectiveness of tyrothricin therapy in staphylococcic infections. The present report deals with the observations made on the resistance of staphylococci to the action of tyrothricin.

Methods. With the exception of one strain (Lederle) all strains of *Staphylococcus aureus* used in this study were recently isolated from patients infected with this microörganism. The strains were stored on blood-agar slants until needed.

A stock solution of tyrothricin* in 95% alcohol was prepared in a concentration of 50 mg per cc. This solution of tyrothricin was added to sterile 10% glucose in distilled water so that the desired concentration was contained in 0.5 cc. The glucose tyrothricin suspension was then added to eleven tubes containing 4.5 cc of veal-infusion broth. The final concentration of tyrothricin in these tubes varied from 0.001 to 1.6 mg per cc. To each series of tubes 0.1 cc of a 12-hour broth culture of the test strain of *Staphylococcus aureus* was added. The final number of organisms present in the inoculated tubes was found to be 10^6 per cc.

The series of tubes were incubated at 37°C for 24 hours. The occurrence of visible growth was noted after 24 hours' incubation. At 24 hours 0.1 cc of each culture was transferred to 5 cc of veal-infusion broth. Following this 0.1 cc was removed from the subcultures in a second transfer. Both series of tubes were incubated for 24 hours and visible growth noted.

Development of Resistant Strains in vitro. Resistant strains were developed *in vitro* by repeated transfers in increasing concentrations of tyrothricin in veal-infusion broth. A series of 11 culture tubes as described above was inoculated with 0.1 cc of a 12-hour broth culture of one of the 3 pathogenic strains of *Staphylococcus aureus.* At 24-hour intervals transfers were made from the tube containing the greatest concentration of tyrothricin and showing full growth to a fresh series of 11 tubes containing tyrothricin. Comparative tests using the original strains and the strains passed through tyrothricin were run simultaneously as described above.

* Tyrothricin was supplied by Dr. John Henderson, Sharp and Dohme Laboratories, and by Dr. Weed Malcolm of the Lederle Laboratories, Inc.

Results. The lowest concentration of tyrothricin required to cause inhibition of growth and killing of each of the 19 strains of *Staphylococcus aureus* was determined. Inhibition was determined by the absence of visible growth in the culture medium containing tyrothricin which had incubated for 24 hours. Killing was determined by absence of growth in both series of subcultures of the medium containing tyrothricin.

In these tests inhibition of growth was produced by a concentration of from 0.024 to 0.2 mg per cc. Twelve of the 19 strains were inhibited by a concentration of less than 0.1 mg. A bactericidal effect was exhibited by a concentration of from 0.024 to 0.8 mg of tyrothricin per cc. Nine strains of staphylococci were both inhibited and killed by identical concentrations of the bactericidal substance, in the remaining 10 strains a somewhat greater concentration of tyrothricin than that causing inhibition was required to produce killing.

These results demonstrate, then, that there is roughly a 10-fold difference in the concentration of tyrothricin necessary to produce inhibition or killing of the various strains of *Staphylococcus aureus.*

Production of Tyrothricin-resistant Strains. Although different strains of *Staphylococcus aureus* vary in their susceptibility to the action of tyrothricin, this variation did not appear to explain entirely the failures encountered in the treatment of local infections caused by this organism. Such a failure occurred in a male, aged 65 years, who had a large ulcer of the lower leg secondary to an inadequate circulation. On institution of tyrothricin therapy the ulcer became sterile and rapid healing took place. After 25 daily applications of tyrothricin in amounts varying from 20 to 100 mg the patient was discharged from the hospital. At this time the ulcer was practically healed, so that it seemed reasonable to expect that ambulatory treatment with tyrothricin would keep the lesion sterile. During the next several months tyrothricin was applied at irregular intervals. Cultures of the ulcer during this period frequently yielded *Staphylococcus aureus* and the ulcer gradually enlarged. At this time the organism was isolated and stored on a plain agar slant for future study. The application of tyrothricin was gradually increased in frequency until it was applied twice daily. In spite of frequent treatment with the bactericidal substance, the cultures remained positive. After 74 days of therapy the organism was again isolated and its resistance to tyrothricin compared to the strain previously isolated from the same patient.

In Table I the results obtained with the 2 strains isolated from the ulcer (Sweeney) are recorded. The original strain showed

definite inhibition of growth with concentrations of tyrothricin greater than 0.006 mg per cc of broth. A bactericidal effect was observed only when the concentration of tyrothricin was greater than 0.05 mg per cc. The organism isolated after 74 days of intensive therapy exhibited a marked increased resistance to the effect of tyrothricin as determined by this test. Inhibition was noted in cultures containing tyrothricin in concentrations of 0.2 mg per cc, and killing occurred with concentrations greater than 1.6 mg. The strain isolated 74 days after the institution of intensive therapy required, then, about 30 and 60 times as much tyrothricin as the original strain to produce the same degree of inhibition and killing respectively.

The above observations led to the attempt to produce *in vitro* strains of *Staphylococcus aureus* which resisted the action of tyrothricin. The results of these studies are included in Table I. Markedly resistant strains were produced after 20 to 28 days' exposure to increasing concentrations of tyrothricin. The original strain (Sweeney) isolated from the patient mentioned above became very resistant to tyrothricin after 20 days' growth in broth containing tyrothricin. Strain Maher showed an increased tolerance to tyrothricin of about 160-fold as determined by bactericidal action. These observations demonstrate, then, that staphylococci may become resistant to the action of tyrothricin both *in vitro* and *in vivo*.

TABLE I.
Production of Tyrothricin Resistant Strains of *Staphylococcus aureus*.

Strain	Days exposure to tyrothricin	Growth	Concentration of tyrothricin in mg per cc											
			0	.001	.002	.006	.012	.025	.05	.1	.2	.4	.8	1.6
Sweeney	0	Visible	+	+	+	0	0	0	0	0	0	0	0	0
		Subculture	+	+	+	+	+	+	+	0	0	0	0	0
	20	Visible	+	+	+	+	+	+	+	+	+	+	0	0
	in vitro	Subculture	+	+	+	+	+	+	+	+	+	+	+	+
	74	Visible	+	+	+	+	+	+	+	+	0	0	0	0
	in vivo	Subculture	+	+	+	+	+	+	+	+	+	+	+	+
Maher	0	Visible	+	+	+	0	0	0	0	0	0	0	0	0
		Subculture	+	+	+	0	0	0	0	0	0	0	0	0
	28	Visible	+	+	+	+	+	+	+	+	+	+	+	0
	in vitro	Subculture	+	+	+	+	+	+	+	+	+	+	+	+
Ram	0	Visible	+	+	+	+	+	0	0	0	0	0	0	0
		Subculture	+	+	+	+	+	+	+	0	0	0	0	0
	27	Visible	+	+	+	+	+	+	+	+	+	+	0	0
	in vitro	Subculture	+	+	+	+	+	+	+	+	+	+	+	0

Discussion. The different strains of *Staphylococcus aureus* associated with clinical infections showed a definite variation in their susceptibility to the action of tyrothricin when measured by either inhibition of growth or actual killing. Although the majority of strains were killed by concentrations of 0.2 mg of tyrothricin per cc, two organisms required a concentration of 0.4 and 0.8 mg of tyrothricin to cause the same effect. This variation in susceptibility of the staphylococcus to the action of tyrothricin is believed to be a factor in determining the successful sterilization of local lesions treated with the bactericidal substance. The organism (Strain 10) isolated from a patient whose infection responded most readily to tyrothricin-therapy was found to be inhibited and killed by a concentration of 0.024 mg per cc. Streptococcal infections in man respond more readily than staphylococcal infections,[5] and by determining the relative susceptibility of numerous streptococcal strains we found that a concentration of tyrothricin as small as 0.0002 mg per cc caused both inhibition of growth and actual killing.

It has been demonstrated that staphylococci which were exposed in broth to increasing concentrations of tyrothricin soon developed a definite increased resistance to the action of the bactericidal substance. That such acquired tolerance may play a rôle in clinical infections was demonstrated in one patient. In this instance large amounts of tyrothricin applied to the local lesion after several months of treatment failed to sterilize whereas early in the course of therapy sterilization was effected readily.

These two factors, then, should be considered in explaining the failures encountered in staphylococcal infections treated with tyrothricin. Other factors believed to be of significance in determining the effectiveness of this form of therapy are the presence of inhibiting substances such as pus, serum, or gram-negative bacteria[5] and the accessibility of the staphylococci to the particles of tyrothricin which are applied.

Conclusions. Various strains of *Staphylococcus aureus* differ in their susceptibility to the action of tyrothricin. It is possible to increase the resistance of staphylococci to tyrothricin by growing the organism in increasing concentrations of the bactericidal substance. In one patient treated with tyrothricin there was a marked increased resistance of the infecting organism after several weeks of therapy.

This work was supported in part by Lederle Laboratories, Inc. Miss Marjorie Jewell and Thelma Maxon gave assistance in these studies.

[5] Rammelkamp, C. H., and Keefer, C. S., unpublished observations.

13560

Relative Susceptibility of Warm Blooded Animals to Ouabain, Cymarin, and Coumingine Hydrochloride.

K. K. Chen and Charles L. Rose.

From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis.

In previous reports[1, 2, 3] from this laboratory, it was shown that the nebulous toad and the spadefoot toad were more tolerant to ouabain, cymarin, and coumingine HCl than the Leopard frog and the tree frog. The work has now been extended to warm blooded animals with the same specimens of the digitalis-like substances, and the results may be presented as further examples of species difference of drug reaction.

Albino mice, albino rats, guinea pigs, New Zealand red rabbits, and cats were employed. The average weights were mice 16.3 g, rats 89.9 g, guinea pigs 334.1 g, rabbits 1.83 kg, and cats 2.38 kg. All drugs were administered subcutaneously, so that the data may be comparable to those already published[3] on frogs and toads by injection into the ventral lymph sac. Solutions of each substance varying from 0.01 to 0.1% were used in most instances except in rats. On account of the low susceptibility of the latter, a 1% solution of ouabain and cymarin was required in order that the volume might not be excessive. Ethyl alcohol was added to ouabain and cymarin to effect a 1% solution—being 9.5 and 57% by volume, respectively. The alcohol was also present in the 0.1% solution of cymarin to the extent of 9.5% by volume. The remaining concentrations and dilutions were all prepared with water.

Following the subcutaneous injection of various doses of ouabain, cymarin, and coumingine HCl, a majority of the animals died within 24 hours, or fully recovered. A few rats succumbed on the second or third day. In any event, the injected animals were observed for one week, so that any delayed deaths, very few in the series, could be recorded. Control animals injected with an equivalent, or twice the amount of alcohol all survived. The protocols are summarized in Table I, and the median lethal doses ($LD_{50} \pm$ standard error) and the approximate ratios of susceptibility are computed and given in

1 Chen, K. K., and Chen, A. L., *J. Pharm. and Exp. Therap.*, 1933, **47**, 295.

2 Chen, K. K., Hargreaves, C. C., and Winchester, W. T., *J. Am. Pharm. A.*, 1938, **27**, 307.

3 Blair, A. P., Hargreaves, C. C., and Chen, K. K., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 209.

Table II. There can be little doubt from the results that cats, rabbits, and guinea pigs are much more susceptible than rats and mice, particularly with reference to ouabain and cymarin. The natural resistance of the rat to strophanthin and digitalis has been known for sometime in accordance with the works of Hatcher[4] and Gunn.[5] Our observations made with different substances are in full agreement with theirs. Since the mouse very frequently follows the rat in response to various drugs, it is not surprising that it, too, possesses natural tolerance to digitalis-like products.

It is interesting to note that coumingine HCl, an alkaloid having a digitalis-like action, confers a lower ratio of susceptibility on mice and rats, being 1:13 and 1:29, respectively, if the cat is taken as

TABLE I.

Acute Toxicity of Ouabain, Cymarin, and Coumingine HCl in 5 Species of Animals by Subcutaneous Injection.

	Ouabain		Cymarin		Coumingine HCl	
Animal	Dose: mg per kg	No. died / No. inj.	Dose: mg per kg	No. died / No. inj.	Dose: mg per kg	No. died / No. inj.
Mouse	4.0	2/8	18.0	4/8	2.0	2/8
	6.2	3/8	22.5	4/8	4.0	2/8
	8.0	4/8	27.5	5/8	6.0	8/8
	12.0	7/8	33.0	6/8	8.0	8/8
	16.0	7/8				
	22.5	8/8				
Rat	27.5	1/5	40.0	0/5	6.0	0/5
	40.0	1/5	50.0	1/5	8.0	5/10
	62.0	1/5	62.0	5/5	10.0	9/10
	80.0	1/5	70.0	5/5	12.0	5/5
	120.0	5/5			14.0	5/5
	160.0	5/5				
Guinea pig	.140	0/5	.275	0/5	.140	0/4
	.180	0/5	.500	3/5	.180	2/5
	.225	3/5	1.000	5/5	.225	4/5
	.275	6/6	2.000	5/5	.275	4/5
					.330	5/5
					.400	5/5
Rabbit	.110	0/5	.225	0/5	.110	0/5
	.180	0/5	.330	2/5	.180	0/5
	.275	3/5	.500	5/5	.275	3/5
	.400	5/5	.600	5/5	.400	5/5
Cat	.100	1/5	.125	0/3	.180	0/5
	.125	4/5	.160	1/6	.225	0/6
	.140	8/11	.200	4/6	.275	2/5
	.160	7/8	.250	5/5	.330	5/5
	.200	5/6			.400	4/4

[4] Hatcher, R. A., *Am. J. Physiol.*, 1909, **23**, 303.

[5] Gunn, J. A., *J. Pharm. and Exp. Therap.*, 1912-13, **4**, 225.

TABLE II.
Ratio of Susceptibility.

Animal	Drug	$LD_{50} \pm$ S.E. mg per kg	Approximate ratio of susceptibility when the cat is unity
Cat	Ouabain	.1119 ± .0158	1
	Cymarin	.1853 ± .0102	1
	Coumingine HCl	.2790 ± .0126	1
Rabbit	Ouabain	.2648 ± .0265	1:2
	Cymarin	.3416 ± .0290	1:2
	Coumingine HCl	.2648 ± .0265	1:1
Guinea Pig	Ouabain	.2205 ± .0110	1:2
	Cymarin	.4760 ± .0571	1:3
	Coumingine HCl	.2003 ± .0160	1:1
Mouse	Ouabain	6.977 ± .986	1:62
	Cymarin	19.78 ± 3.56	1:107
	Coumingine HCl	3.560 ± .498	1:13
Rat	Ouabain	75.08 ± 12.76	1:671
	Cymarin	52.90 ± 2.64	1:285
	Coumingine HCl	8.118 ± .395	1:29

unity. This appears to agree with the response of the spadefoot toad which is much more resistant to ouabain and cymarin than to coumingine.[3] Ouabain and cymarin are monosides of the aglycones, ouabagenin and strophanthidin, respectively. Both of these aglycones are derivatives of cyclopentanophenanthrene with a side-chain of unsaturated lactone.[6, 7, 8] Coumingine, on the other hand, has apparently a different configuration.[9] The deviation in chemical structure of coumingine from that of ouabain and cymarin may account for the relatively lower resistance of mice and rats to its toxic action.

It has been suspected sometimes that toads are resistant to digitalis-like substances, because they immunize themselves with bufagins and bufotoxins of their own venom, both of which belong to the digitalis class. Such a possibility may be excluded now for several reasons: first, bufagins and bufotoxins are not present in toads' circulation;[10] secondly, the same substances, having small molecular

6 Fieser, L. F., and Newman, M. S., *J. Biol. Chem.*, 1936, **114**, 705.

7 Jacobs, W. A., and Elderfield, R. C., *Science*, 1934, **80**, 533.

8 Paist, W. D., Blout, E. R., Uhle, F. C., and Elderfield, R. C., *J. Org. Chem.*, 1941, **6**, 273.

9 Ruzicka, L., Dalma, G., and Scott, W. E., *Helv. Chim. Acta*, 1941, **24**, 63.

10 Chen, K. K., and Chen, A. L., *Arch. internat. de pharmacodyn. et de thérap.*, 1934, **47**, 297.

weights, are unlikely to induce an immunological process like proteins; and thirdly, rats and mice, devoid of any digitalis-like principles in their body secretions, are comparable to toads in their tolerance to cymarin, ouabain, and coumingine HCl.

In the assay of digitalis-like principles, cats and frogs are most frequently used. When the results in cats are compared with those of frogs published in a previous communication,[3] cats appear to be more susceptible. However, part of the difference may be due to temperature variations, for the frogs were studied in a water bath maintained at 20°C, while the cats were investigated at a room temperature of 24.5°C.

Summary. 1. The acute toxicity of ouabain, cymarin, and coumingine hydrochloride has been compared by subcutaneous injection in cats, rabbits, guinea pigs, rats, and mice. 2. Both mice and rats are much more tolerant to ouabain and cymarin than cats in the ratios of 62:1 to 671:1. Regarding the alkaloid, coumingine HCl, which also has a digitalis-like action, their resistance is not so marked, being in the ratios of 13:1 to 29:1. 3. Guinea pigs and rabbits, like cats, are highly susceptible to ouabain, cymarin, and coumingine HCl.

13561

Preparation of Living Nuclei from Hen Erythrocytes.

MICHAEL LASKOWSKI.* (Introduced by L. S. Palmer.)

From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul, Minn.

The early attempts to separate the nuclei from avian erythrocytes resulted in obtaining an amorphous mass of nuclear material. Warburg[1] was the first to isolate the separate nuclei. He used a freezing-melting technic. The same method was used by Miyake.[2] The disadvantages of this method are: partial agglutination and damage of nuclei, incomplete hemolysis and the necessity of further separation.

The method to be described leads to a stable suspension of free

* General Mills, Inc., Research Fellow.

Paper No. 1959 Scientific Journal Series, Minnesota Agricultural Experiment Station.

1 Warburg, O., *Z. physiol. Chemie*, 1910, **70**, 413.

2 Miyake, M., *Keijo J. Med.*, 1933, **4**, 247.

nuclei which probably have been less damaged than in the freezing technic. The method is based on the hemolysis of hen erythrocytes with lysolecithin in neutral saline solution, washing the nuclei with saline and resuspension in saline.

Lysolecithin was prepared as follows: Lecithin emulsion (0.5 g per 20 ml) was made in phosphate buffer pH 7.0-7.1 (crude lecithin recovered from cadmium salt of student preparation was used). The lecithin emulsion was ground with poison glands of 100 bees (lecithinase A), incubated during 24 hours at 37°C and filtered through a Berkefeld filter.

Chicken blood was obtained by cardiac puncture, according to the method of Sloan and Wilgus.[3] Citrate was used to prevent clotting. The blood was centrifuged at a speed just sufficient to sediment the erythrocytes and leave the bulk of the leucocytes in suspension. The plasma with suspended leucocytes and the top layer of erythrocytes were pipetted off. Isotonic saline solution was added to the rest of the erythrocytes and the procedure was repeated several times (5-6). Finally, the erythrocytes were centrifuged to close packing, the pipette introduced to the bottom of the centrifuge tube, and the lower layer of erythrocytes pipetted into a second centrifuge tube and suspended in saline. Microscopic examination of this suspension showed only occasional leucocytes.

To 30-40 ml of the erythrocytes suspension, corresponding approximately to the same volume of original blood, about 5-8 ml of hemolysing solution were added. The hemolysis was followed by observation of color in the liquid and controlled by microscopic examination. After the hemolysis was completed, usually 20 to 40 minutes at room temperature, the nuclei suspension was centrifuged, washed several times (5-6) with saline, and resuspended in the volume of saline approximately corresponding to the volume of the original blood.

The nuclei suspension thus obtained had a very faint yellow color. Examined under the microscope the free nuclei appeared smaller and more compact than the nuclei within the intact erythrocytes. The free nuclei stained readily with the aqueous methylene blue.

The nuclei suspension was stable when stored during 2 weeks in the refrigerator. When a few drops of nuclei suspension were poured into 3-4 ml of water almost immediate agglutination occurred. The nuclei suspension was stable in 0.1 M KH_2PO_4, but not in 0.1 M Na_2HPO_4. Microscopic examination in Na_2HPO_4 solution showed the nuclei with partially broken membranes and with the nuclei con-

[3] Sloan, H. J., and Wilgus, H. S., Jr., *Poultry Science*, 1930, **10**, 10.

tent streaming out from the punctured membranes (aqueous methylene blue staining). In saturated NaCl solution the nuclei formed a transparent gel.

A few preliminary experiments showed that the nuclei thus obtained respire. Warburg's direct method was used. In saline buffered with phosphate to pH 7.3 the Q_{O_2} value was around 0.2. It did not change appreciably after 2 days' storage in the refrigerator. When glucose was added to the medium the value of Q_{O_2} rose to about 0.3. The respiration varied at different pH values (phosphate buffers) as follows:

pH	Vol.	$cmmO_{2/h}$	Qo_2
6.0	1.0 ml	2.83	0.094
6.5	1.0 ml	3.87	0.13
7.0	1.0 ml	6.33	0.21
7.5	1.0 ml	5.83	0.19

Agglutination of the nuclei with water did not significantly influence the respiration. In saturated sodium chloride the respiration first increased then diminished and stopped. The temporary increase could be regarded as bringing into contact the enzymes and substrates of nuclei after the internal structure was disrupted.

The respiration of the original erythrocyte suspension in the identical conditions gave Q_{O_2} values between 0.15 and 0.22, which is about one-third of the values found in Warburg's laboratory[4] for goose erythrocytes. Addition of glucose to the medium did not increase the respiration materially.

When erythrocytes were hemolysed within the Warburg apparatus either with lysolecithin or with water a significant decrease of respiration occurred. Judgment is withheld, until more evidence can be collected, as to whether this decrease is due to the partial damage of nuclei or only to the disruption of the connection between the nuclei and the cytoplasm.

The same method of preparation can be applied to the erythrocytes of mammalian embryos.

[4] Negelein, E., *Biochem. Z.*, 1925, **158**, 121.

13562 P

Transmission of Virus of Infectious Tracheitis in Chicken by Contaminated Egg Shell.

I. J. Kligler and M. Perek.

From the Department of Hygiene and Bacteriology, Hebrew University, Jerusalem.

An infectious disease hitherto unobserved in Palestine was reported among chicks from different poultry farms. Symptomatology resembled infectious laryngotracheitis[1]; it differed from this infection by its mild course, absence of fatalities and of any noticeable effect on development. The disease is milder in young than in old chicks.

Mucus from the trachea of infected chickens was triturated with sand, diluted with nine parts saline and divided into two parts: one was used for inoculation, the other was passed through Seitz filter and the filtrate inoculated in the same manner as the unfiltered material. The results demonstrated that the causative agent was a filterable virus. Cultures of the filtrate in a variety of media remained sterile. The virus was recovered from the lung, but not from the liver, ovary or uterus.

Infection was most readily produced by dropping infected material into the trachea, also by intraperitoneal or intrathoracic inoculation. The incubation period varied with the age of chicks and the concentration of virus. One-week-old chicks showed the first symptoms of infection after 5 days; in chicks 2 weeks old, the incubation period was 9 to 24 days; in 6-8-week chicks the incubation period varied from 11 to 42 days. The infection spread quickly by contact. The time of exposure before appearance of symptoms (8-10-week chicks) ranged from 22 to 64 days. The virus could be demonstrated in the tracheal secretion in 1:1,000,000 in unfiltered and in 1:10,000 in filtered fluid.

Experiments demonstrated that the virus was transmitted on the surface of the egg shell.

1. Eggs were obtained from the infected farm and incubated. Of 110 eggs 40 chicks hatched. After 37 days 3 chicks developed the disease and two weeks later 4 more succumbed. The experiment was carried out under strict conditions of isolation so that no contact with any other source of infection was possible. The virus was recovered from the diseased chicks.

---

[1] *a.* Kernohan, G., *Univ. of Cal. Agr. Exp. Sta. Res. Bul.*, 494, 1930; *b.* Hinshaw, W. R., *Univ. of Cal. Agr. Exp. Sta. Res. Bul.*, 520, 1931; *c.* Beach, J. R., *Report of the Sixth World's Poultry Congress*, 1936; *d.* Beaudette, F. R., *Poultry Science*, 1937, **16**, 103.

2. A second lot of 105 eggs was obtained from the same source. These eggs were immersed in a 1:1000 solution of $KMnO_4$ for 15 minutes, washed with $NaHSO_3$, then with sterile water, dried and placed in incubator, which was thoroughly washed and fumigated with formaldehyde. Thirty chicks hatched and developed normally during 61 days' observation.

3. 109 eggs from the same source were incubated as above. Forty-five chicks hatched. At the end of one week 4 chicks showed symptoms and in five weeks 25 chicks developed the disease.

4. Of 104 eggs from the same infected source treated as in Exp. 2, 61 hatched. During 42 days there was no sign of infection.

5a. Eggs were obtained from an uninfected farm. These were infected by painting the shells with the mucus from the trachea of an infected chick. Of 48 eggs, 18 chicks hatched; 8 chicks showed symptoms after 5 days and 3 more by the 10th day.

5b. 50 eggs were treated as in 5a, the mucus diluted 1:10 in saline. Only 9 chicks hatched, 2 showed signs of the disease after 5 days and 3 more by the 10th day.

6. The infected mucus was diluted 1:10 in saline and the suspension sprayed on the surface of eggs from an uninfected source. Of 48 eggs 21 chicks hatched. After 18 days 3 chicks showed typical symptoms and were removed; 10 days later 3 more chicks developed the infection.

7. In the experiments with artificially infected eggs, sets of 2 eggs were removed at the time of incubation, and after 8, 14, 18, and 19 days respectively. The surface of each egg was washed with saline and 0.5 cc of the washings inoculated into the trachea of 2-4-week-old chicks; all chicks developed typical infection within 13 to 21 days. These tests show that the virus dried on the shell was infective during the entire period of incubation. No virus could be demonstrated in dead embryos from eggs artificially infected on the surface, or from eggs from an infected source.

8. Eggs whose shells were artificially infected were divided into two sets. One set was washed with saline, the other was first treated with permanganate and sulphite as above and then washed with saline. The respective saline washings were then inoculated into the trachea of 6-week-old chicks. Those receiving the control washings developed the disease in 11 days, while those receiving the washings after the eggs had been treated with permanganate remained well during a period of observation of 33 days.

9. These experiments were repeated under farm conditions. In the infected farm untreated eggs always yielded about 3-5% infected

chicks. Of 2140 eggs washed with permanganate (average hatch 66%) no infections were noted during 4-6 weeks. However, as soon as these chicks came into contact with existing flocks, the disease spread rapidly among them. When chicks hatched under the same conditions were kept away from infected stock they developed without infection.

10. A total of 26,636 eggs washed with permanganate and sulphite gave an average hatch of 69.1%, showing that treatment has no damaging effect on eggs.

13563

Comparative Effects of Horse Serum, Horse Serum Albumin and Horse Serum Globulin in Experimental Shock.*

HARRY A. DAVIS AND A. G. EATON.

From the Departments of Surgery and Physiology, Louisiana State University School of Medicine, New Orleans.

Previous reports have been made regarding the effects of the intravenous administration of bovine serum albumin to man[1] and to lower animals.[2] The present communication is concerned with the intravenous use of horse serum, horse serum albumin, and horse serum globulin as blood replacement fluids in experimental shock.

Materials. Normal horse serum from healthy horses was used. The albumin and globulin fractions were separated from the horse serum by the method which we have described previously.[1] From analysis, the whole serum contained 9.2 g % of total proteins, 4.0 g % of albumin and 5.2 g % of globulin. After separation, the horse serum albumin solution contained, from analysis, 3.7 g % of albumin. The serum globulin solution contained 5.4 g % of globulin. The experimental animals were healthy dogs weighing from 3.2 kg to 6.8 kg.

* Since the completion of this investigation on bovine and horse serum albumin, our attention has been drawn to two preliminary reports of work along similar lines. No details of methods used or data are given in these reports.[3,4]

1 Davis, H. A., Eaton, A. G., and Williamson, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 96.

2 Davis, H. A., Eaton, A. G., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 20.

3 Janeway, C. A., and Beeson, P. B., *J. Clin. Invest.*, 1941, **20**, 435.

4 Keys, A., Taylor, H. L., and Savage, G. M., *J. A. M. A.*, 1941, **117**, 62.

Methods. Light ether anesthesia was used in all of these experiments. The blood pressure was recorded in the usual manner from a cannula inserted into the right carotid artery. Shock was produced by graded hemorrhage from the carotid artery. The sera were administered by gravity through a cannula inserted into the right femoral vein in amounts equal to the quantity of blood removed from each animal. No preliminary cross-matching of the sera with the blood of the prospective recipient animal was carried out.

Results. Whole Serum. The intravenous administration of whole horse serum caused a rise in blood pressure of dogs subjected to severe hemorrhage. The rise in blood pressure was maintained and all of the animals recovered (Table I). Mild temporary dyspnoea was observed in these animals during and for a short time following the injection.

Serum Globulin. Serum globulin solutions caused marked dyspnoea in all of the animals. One animal died 12 hours after the injection. At autopsy, the tissues of this animal revealed subcutaneous, subserosal, subpleural, subepicardial and subendocardial hemorrhages. The liver was congested and the spleen was contracted. The remaining animals, which survived, were noticeably sick and listless for several days. The rise in blood pressure following the injection of serum globulin solutions was not as pronounced as that following the injection of whole serum or serum albumin solutions (Table I).

TABLE I.

Comparative Effects of Horse Serum, Horse Serum Globulin and Horse Serum Albumin in Shock.

Dog wt, kg	C.B.V., cc	Amt. blood removed, cc	Solution inj., cc	Blood pressure: Initial mm Hg	After hemorrhage mm Hg	After inj. mm Hg	Result
			H.S.*				
4.4	396	180	180	130	30	140	Mild dyspnea, recovery
4.8	432	185	185	110	17	108	" " "
6.0	540	235	235	155	25	150	" " "
			H.S. globulin				
5.2	468	195	195	120	23	93	Severe " "
3.6	324	165	170	155	35	120	" " "
5.6	504	220	220	150	35	115	" " "
4.4	396	185	185	170	40	120	Death 12 hrs later
			H.S. albumin				
6.8	612	210	205	140	20	130	No dyspnea, recovery
3.2	288	100	100	137	50	135	" " "
4.0	360	150	150	135	40	110	" " "
4.8	432	185	185	152	30	150	" " "
3.8	342	110	110	100	26	80	" " "
4.2	378	190	190	140	30	138	" " "

*Horse serum.

Serum Albumin. The intravenous injection of serum albumin solutions resulted in a rise of blood pressure to almost the initial level. The rise in blood pressure was maintained and all of the animals recovered without after-effects (Table I). No dyspnoea or other anaphylactic phenomena were observed in this group of animals.

Discussion. It would appear that normal horse serum is less toxic to dogs than is bovine serum.[2] The toxic effects following the injection of horse serum globulin are similar to those observed with bovine serum globulin.[2] Horse serum albumin, like bovine serum albumin, does not appear to be toxic to dogs, inasmuch as it is able to raise and maintain the blood pressure of dogs subjected to severe hemorrhage. In these experiments the concentration of serum globulin was approximately the same in the whole serum and in the globulin solution obtained after separation. Despite this fact, serum globulin was more toxic when injected alone than when administered with the other constituents of whole serum. This suggests the possibility that some substance in whole horse serum, *e. g.,* serum albumin, tends to diminish the toxicity of the globulin when whole horse serum is injected.

Summary. Horse serum albumin solution is effective in raising and maintaining the blood pressures of dogs subjected to severe hemorrhage. Equally effective is whole serum from the horse. However, horse serum globulin solution is much less effective in this respect and appears to be more toxic.

13564

The Pubertal Increase in Response of Accessory Sex Organs to Steroid Hormones.*

HANS SELYE AND S. ALBERT.

From the Department of Anatomy, McGill University, Montreal, Canada.

The fact that age plays an important rôle in determining the response of the accessory sex organs to stimulation by testoid (or "androgenic") compounds has previously been emphasized on the basis of experiments on female rats. It could be shown that testo-

* The expenses of this investigation were defrayed by the Blanche E. Hutchinson Fund of McGill University and the steroid compounds were kindly donated by Drs. E. Schwenk and G. Stragnell of the Schering Corporation of Bloomfield, N.J.

sterone inhibits the development of the uterus during the first days of life, while at a later term it causes uterine enlargement.[1] More recently Hooker[2] noted that in male rats, castrated at birth, the smallest dose of testosterone necessary to elicit recognizable enlargement of the seminal vesicles, decreases suddenly when the animals reach the pubertal age, that is about 40 days. The experiments to be reported here indicate that the effect of puberty upon the sensitivity to various testoid compounds is dependent upon their chemical structure.

The results of our experiments are summarized in Table I. Each figure in italics represents the average value of a group of 6 rats, the range being indicated in brackets. In every case the animals were castrated on the day before treatment was begun. The daily dose of the finely ground steroids [Δ^5-androstene-3(β),17(α)-diol and 17-ethinyl testosterone] was suspended in 0.2 ml peanut oil and administered in 2 subcutaneous injections (morning and night) on 10 consecutive days. The animals were sacrificed on the 11th day and their organs weighed after fixation in Heidenhain's Susa mixture.

It appears that in the prepubertal animals (weighing less than 60 g at the beginning of the experiment) ethinyl testosterone causes much more pronounced enlargement of the seminal vesicles, prostates and

TABLE I.
Effect of Δ^5-androstenediol and Ethinyl Testosterone on Pre- and Post-pubertal Castrate Male Rats.

Treatment	Mg per day	Body weight in g		Seminal vesicles in mg	Ventral prostate in mg	Middle prostate in mg	Preputial gland in mg	Coagulating gland in mg
		Initial	Final					
Δ^5-Androstenediol	2	*47* (40-55)	*90* (85-100)	*14* (11-18)	*36* (29-44)	*18* (14-25)	*47* (31-78)	*4* (3-6)
Ethinyl Testosterone	2	*48* (40-60)	*92* (80-110)	*43* (36-51)	*55* (31-64)	*38* (32-46)	*29* (15-56)	*11* (9-13)
Δ^5-Androstenediol	6	*120* (105-140)	*153* (130-180)	*130* (82-189)	*101* (90-116)	*83* (71-110)	*80* (55-105)	*29* (20-45)
Ethinyl Testosterone	6	*119* (100-145)	*126* (100-150)	*102* (81-126)	*87* (71-109)	*67* (57-74)	*46* (24-67)	*24* (18-31)
Δ^5-Androstenediol	10	*46* (40-58)	*86* (75-100)	*16* (12-19)	*39* (27-48)	*21* (16-23)	*62* (55-72)	*6* (2-11)
Ethinyl Testosterone	10	*46* (40-58)	*88* (80-100)	*79* (62-91)	*55* (46-70)	*46* (43-49)	*46* (30-59)	*16* (9-21)
Δ^5-Androstenediol	10	*87* (75-95)	*127* (100-150)	*165* (90-196)	*97* (69-126)	*73* (61-84)	*109* (61-152)	*35* (26-39)
Ethinyl Testosterone	10	*87* (75-95)	*130* (120-150)	*79* (65-99)	*78* (60-99)	*50* (48-49)	*53* (32-73)	*22* (16-32)

[1] Selye, Hans, *Endocrinology*, 1940, **27**, 657.

[2] Hooker, Charles W., *Endocrinology*, 1942, **30**, 77.

coagulating glands than Δ^5-androstenediol, while the reverse is true of the post-pubertal castrates. The preputial glands, which are particularly sensitive to androstenediol at any age, do not show this change in sensitivity as clearly as the other accessory sex organs. However, even their responsiveness at puberty increases more markedly to androstenediol than to ethinyl testosterone.

Summary and Conclusions. Experiments on the rat indicate that in prepubertal castrates ethinyl testosterone stimulates the seminal vesicles, prostates and coagulating glands more markedly than an equivalent dose of Δ^5-androstenediol, while the reverse is true in post-pubertal castrates. It appears that at puberty the sensitivity of the accessory sex organs increases only with regard to steroid compounds of a certain chemical structure. This fact must be kept in mind when the hormonal potency of various testoid substances is to be compared.

13565

Pantothenic Acid Absorption in Pernicious Anemia.

CURTIS E. MEYER, IRVING F. BURTON AND CYRUS C. STURGIS.

From the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan, Ann Arbor, Mich.

The most generally accepted view regarding the etiology of pernicious anemia is that the nervous manifestations of the syndrome result from the lack of some other factor than that required for hematopoiesis. The anemia and the neurological lesions frequently develop independently, since one may be present for long intervals before the other. The knowledge that this may occur has led to the acceptance, by a majority of observers, of the dual deficiency theory of the etiology of the disease.

Considerable attention, especially in recent years, has been directed toward the possibility that a deficiency of one or more vitamins might be responsible for the neural disturbances. These observations, together with the reported occurrence of the neuropathology of the spinal cord in chicks receiving a diet deficient in pantothenic acid,[1] prompted the work herein reported. Like thiamin,[2]

1 Phillips, P. H., and Engel, R. J., *J. Nutrition*, 1939, **18**, 227.

2 Field, H., Jr., Robinson, W. D., and Melnick, D., *Ann. Int. Med.*, 1940, **14**, 588.

pantothenic acid is alkali-labile and hence possibly might be destroyed in the intestinal tract of achlorhydric patients. Since study of the urinary excretion of the vitamins has proven of value in certain deficiency diseases, the following experiment was carried out. The daily pantothenic acid excretion of patients with pernicious anemia was compared to that of healthy subjects both with and without the previous administration of 100 mg of calcium pantothenate.

Experimental. From 2 to 8 individual 24-hour urine specimens from each of 7 patients with Addisonian pernicious anemia and one sample from each of 7 healthy medical students and older laboratory workers were collected and preserved with toluene. Both groups received nutritionally good diets during the course of the experiment although some of the patients did not eat normal quantities during the first few days. Pantothenic acid was determined by the method of Pennington, Snell and Williams,[3] with assays made at 4 levels. When a supplement of acid-autoclaved urine was added to the medium as recommended by these investigators, excellent agreement between the various assay levels was obtained.

In Table I are listed the values observed. The amounts found in the control specimens are somewhat greater than reported by Pelczar and Porter,[4] who observed an average excretion of 3.8 mg with a range of 1.46 to 6.79 mg. The average output of the patients, 4.4 mg per day, is not significantly lower than that noted for our control

TABLE I.
Pantothenic Acid Excretion.*

Pernicious anemia patients					Controls		
No.	Determinations†	Range, mg	Avg, mg	After 100 mg dose,‡ mg	No.	Daily excretion, mg	After 100 mg dose,‡ mg
1	8	4.9-8.0	6.8		1	5.7	16.5
2	2	4.2-4.9	4.5		2	4.4	
3	3	4.2-4.7	4.4		3	5.5	
4	5	2.7-5.1	3.9	14.0	4	5.9	13.5
5	5	2.5-7.3	4.9	15.4	5	7.1	30.6
6	5	2.7-5.7	3.8	9.7	6	8.1	20.8
7	4	2.0-3.4	2.7	13.5	7	5.9	10.9
	Avg		4.4	13.1		6.1	18.4

*Mg per 24-hour specimen.
†Each determination was made on a separate specimen.
‡100 mg of calcium pantothenate[1] was given orally.
[1] Merck and Co. generously supplied the calcium pantothenate.

[3] Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **135**, 213.

[4] Pelczar, M. J., and Porter, J. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 3.

subjects and certainly as high as others have reported.[4] That the lower excretions were probably due to a previously low intake of the compound is strongly suggested by the fact that in every case where there was an appreciable difference in the range of the daily excretion, the lower values were observed at admission of the patient, with a progressive rise while on the hospital diet. A typical case was patient No. 5 whose consecutive daily excretions were 2.5, 3.0, 5.6, 6.2, and 7.3 mg. This increase was not due to the parenteral liver extract, for analyses of the preparation indicated that he received less than one mg from this source during the whole course of treatment.

Further evidence that the excretion of pantothenic acid by the patients was not limited by impaired absorption was obtained by the oral administration of calcium pantothenate. Following 100 mg doses of the salt, the patients and controls excreted comparable amounts, as shown in the table.

This study is being extended to include other water-soluble vitamins.

Conclusions. In patients with pernicious anemia, the daily excretion of pantothenic acid both before and after administration of 100 mg of its calcium salt was slightly but probably not significantly less than that of healthy individuals studied in the same manner. This indicates there is no impairment of absorption of this compound in patients with pernicious anemia with which is always associated achlorhydria. The possibility must be considered, however, that the lack of hydrochloric acid may make the pantothenic acid in food less available for absorption. This is undergoing further study.

13566 P

Electrocardiographic Changes Associated with Acute Pancreatitis.

J. GOTTESMAN, D. CASTEN AND A. J. BELLER. (Introduced by H. L. Jaffe.)

From the Laboratories and Surgical Services of the Hospital for Joint Diseases, New York City.

We have had occasion to observe a series of cases of acute pancreatitis, in which the signs of upper abdominal peritoneal irritation were associated with elevated blood amylase and abnormal electro-

cardiograms. Serial electrocardiograms taken throughout the course of the disease in those who survived showed a return to a normal pattern as the clinical signs and symptoms of the disease abated, and as the elevated blood amylase became normal. The transient character of the electrocardiographic changes suggested to us that they were caused by the acute pancreatitis rather than by any intrinsic cardiac pathology. The following abstract of one of the cases is illustrative of the various changes observed during the course of the disease.

E. B., colored, male, 38, admitted December 5, 1941, because of abdominal pain, nausea and vomiting of 5 days' duration following a drinking spree. Examination showed a dehydrated, acutely ill patient, complaining of upper abdominal pain. The abdomen was distended, and was markedly tender throughout the epigastrium and the left upper quadrant. The entire abdomen was rigid. The clinical diagnosis of acute pancreatitis was made.

The course is outlined below :

Date	Condition of patient	Electrocardiogram	Blood Amylase	Other findings
12/5	Acutely ill Temp. 100, Pulse 80, abdomen tender	Depressed ST segment in leads 2 and 3. Diphasic T waves in leads 1, 2 and 3. Myocardial damage.	410	WBC 11,200.
12/8	Condition unchanged. Temp. 100, Pulse 80.	T 1 inverted T 2 diphasic RT 4 depressed Myocardial damage	374	
12/11	Vomiting persists. Tenderness in left upper quadrant.	T 1 iso-electric T 2 low T 4 inverted Myocardial damage	315	
12/19	General condition improved. Patient ambulatory.	All changes previously noted have disappeared. Electrocardiogram normal.	303	Blood sugar 64 N.P.N. 19 Chol. 164 Chol. esters 68%
1/15/42	Patient well. No complaints.		254	

A study was undertaken on dogs to determine whether these changes could be duplicated in experimental acute pancreatitis.

Method of Study. Under intravenous nembutal anesthesia the accessory pancreatic duct in dogs was exposed, 5 cc of a 12% solution of sodium taurocholate was injected into the pancreas and the duct was then ligated. Pancreatic congestion was noticed immediately, and a fatal pancreatitis was produced, death occurring after 12 to 15 days. Autopsy revealed the presence of acute pancreatitis with abscess formation.

Electrocardiograms were taken preoperatively and at 2- to 3-day

intervals postoperatively until the animal died. Control electrocardiograms were taken in dogs similarly anesthetized but not operated upon.

The electrocardiograms in the operated dogs showed distinct aberrations from the normal preoperative tracings.

Protocol Dog 4 operated 1/13/42. Preoperative electrocardiogram—sinus arrhythmia.

1/14/42 Electrocardiogram—inversion of the T wave in lead 1.
1/16 " inversion of the T wave in lead 1.
1/19 " inversion of the T wave in lead 1.

Changes became progressively more marked, indicative of increasing myocardial damage.

The changes were interpreted in some instances as indicative of myocardial damage and in others as showing evidence of coronary closure with infarction. There was no constancy in the electrocardiographic patterns nor did they always correspond to the definite electrocardiographic changes observed clinically in heart disease.

The exact mechanisms underlying these changes are being further studied. Observations are also being continued on the association of electrocardiographic changes with other acute intraäbdominal lesions.

Summary. Clinical observations have demonstrated the association of acute pancreatitis with transient electrocardiographic changes. We were able to obtain similar changes in dogs with experimentally induced pancreatitis.

13567

Relationship Between Body Size and Metabolism.

F. W. Weymouth, John Field, II, and Max Kleiber.

From the Department of Physiology, Stanford University, and the College of Agriculture, University of California, Davis.

Recently Kleiber has studied the relation in liver slices of oxygen consumption to body weight in rats, rabbits, and sheep.[1] It was shown that the Q_{O_2} was inversely proportional to a fractional power of the body weight, so that plotted on a log log grid the data gave a straight line represented by the formula

$$Q_{O_2} = 5.26\ W^{-0.24}$$

[1] Kleiber, Max, Proc. Soc. Exp. Biol. and Med., 1941, **48**, 419.

where Q_{O_2} is in mm^3/mg dry tissue/hour and W is the body weight in kilos.

It was concluded that "the factors which determine the metabolic level *in vivo* seem still to be present in the surviving tissues cut out of the organism."

Subsequent determinations of tissue respiration and observations on the relation of total metabolism to body size justify an additional note on this subject.

A recalculation of values from the original individual records of Kleiber gives a coefficient of correlation between log Q_{O_2} and log body weight of –0.783 and a coefficient of regression of –0.228 with a standard error of ±0.017. The formula thus becomes

$$Q_{O_2} = 5.28\ W^{-0.228*}$$

Field has since obtained data on respiration in kidney and brain slices for the rat and rabbit. Although covering only two species, the regression obtained is very similar, the average being –0.199. A value as low as this might be expected to occur by random sampling in data similar to Kleiber's about once in 10 times so that the difference can hardly be considered as significant. It is thus apparent that other tissues behave in a manner very similar to liver slices. This strengthens the view previously expressed by Kleiber.

On the other hand it is interesting to compare the exponent with that required by the surface area "law", 0.333. The chances that a value as high as 0.333 could occur from sampling of Kleiber's material is less than one in 10^{+8}. There can be no doubt that the present data differ significantly from that required by the surface area "law".

We have subsequently noted that the weight-specific rate of heat production of intact animals of these species bears the same relation to the body weight as that stated above for the tissues *in vitro*. The data given in Table I were taken from Benedict[2]; the body weights

* In Article 13340, Body Size and Metabolism of Liver Slices *in vitro*, PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 419, 18 instead of 42 kg was used as mean weight of the sheep. The correct weight leads to the following relation between mean Q_{O_2} of liver slices from rats, rabbits, and sheep and their respective mean body weights:

$$\log Q_{O_2} = 0.735 - 0.21 \log W$$
$$\text{or } Q_{O_2} = 5.43\ W^{-0.21}$$

(The omission of the minus sign of the exponent in the original article is a typographical error). Fortunately the error had no significant effect on the conclusions.

[2] Benedict, Francis G., *Vital Energetics*, Carnegie Institution, 1938, p. 131 and 175.

TABLE I.
Heat Production by Animals of Different Body Weights.*

Animal	Body wt, kg	Heat production Cal./kg/day
Rat	0.4	82.0
Rabbit	2.6	44.5
Sheep	45.0	25.5

*Data from Benedict Table 2, p. 131, and Table 4, p. 174. These two tables do not check. I have used figures from Table 2, except for the heat production of the sheep, which is given as 35.50, obviously a misprint for 25.50, as may be seen by comparing with Fig. 35, p. 142.

differ from those given in Kleiber's paper and presumably represent different breeds of these species. The log log plot of heat production (Cal./kilo/day) on body weight (kg) gives a straight line represented by the formula

$$H = 6.87\ W^{-0.243}$$

This power of the body weight might have occurred by random sampling in Kleiber's data once in 3 or 4 times, so that the difference cannot be considered significant. The other constant cannot be compared because of the different units employed and the fact that one is dry, and the other, wet weight. We thus see that there is complete agreement between the weight-specific heat production rate in the intact animal and the respiratory rate in the excised tissues of the same series of animals. This has not, as far as we know, been pointed out before. The relation here presented supports the opinion expressed in the previous paper.

It is clear that we must envisage a mechanism of respiratory regulation which will account for the following observed facts, a far from easy task.

a. The respiratory rate (Q_{O_2}) varies widely; the greatest range is from tissue to tissue in a particular animal (150-fold between extremes, kidney and blood, in the rat[3]), and less for a particular tissue from animal to animal with change of size (4-fold in the present data from rat liver to sheep liver, 10-fold from the mouse to the elephant, if all tissues parallel the weight-specific rate[2]). In addition there are apparently qualitative as well as quantitative differences in the enzyme systems involved (Commoner[4]).

b. The control is not immediately systemic, for example through the circulation by regulation of the supply of oxygen or of some substrate, since the relation to body weight of oxygen consumption

[3] Field, John, II, Belding, H. S., and Martin, A. W., *J. Cell. Comp. Physiol.*, 1939, **143**, 14.

[4] Commoner, B., *Biol. Revs.*, 1940, **15**, 168.

from animal to animal is the same for tissue slices in an atmosphere of oxygen as for the total metabolism of the intact organism.

c. The control is intrinsic in the sense that the relation to body weight of the respiration of the excised tissue slices parallels the metabolism of the intact animal, at least during the one or 2 hours required by the Warburg technic. If the control is ultimately central, as by some hormonal regulation of the concentration of respiratory enzymes, the peripheral "set" involved must have a large measure of inertia, as it is not readily altered by excision and the disruption of all systemic channels of control.

d. Through a striking range the intensity of respiration is regulated between tissue and tissue and from animal to animal so as to present the following picture: The relative intensity of respiration is proportional to the relative body weight of the animal, while the tissues preserve their relative relations. As a result the plot of the log oxygen consumption on the log body weight (thus placing both on a relative basis) yields a family of parallel straight lines, one for each tissue. This conclusion is necessitated not only by the relation shown above for the Q_{O_2} of kidney, liver and brain but also by the fact that the total metabolism of a series of animals parallels the Q_{O_2} of any one of the tissues for these same animals.

13568

Agglutination of Rabbit Leucocytes by *Staphylococcus aureus* Toxin.

JULIA T. WELD AND LUCY C. MITCHELL.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

Several years ago, while testing streptococcal-extract toxin[1] for leucocidin by the Neisser-Wechsberg technic, one of us noted agglutination of the leucocytes in some of the tubes. Such agglutination appeared only on dilution of the toxins.

Recently a similar phenomenon was observed with staphylococcal toxin. This paper deals with a method developed for testing of staphylococcal toxin for these leuco-agglutinins.

Methods. Most of this work was carried out with the Wood strain

[1] Weld, Julia T., *J. Exp. Med.*, 1934, **59**, 83.

of staphylococcus. However, similar results have been obtained with several other strains.

The medium used was a phosphate-buffered veal-infusion broth,[1] with proteose-peptone, containing 1-20,000 phenol-red indicator (Merck). This medium was used for the growth of the organisms in the preparation of the toxins and, without peptone, for the suspension of the leucocytes and the dilution of the toxins used in the tests.

The organisms were grown for 4 or 5 days in 50 cc Erlenmeyer flasks containing 25 cc of medium and 0.5 g of cellophane.[2] The cultures were then centrifuged, filtered at pH 8 through Berkefeld V filters and stored at pH 7.2 in the ice box, usually under vaseline seals.

The leucocytes were obtained from young rabbits weighing 1500 to 2000 g following the intrapleural injection of 20 cc of saline containing 5% aleuronat and 3% starch. Eighteen hours after injection the animals were bled to death from the carotid and the pleural exudates taken up in 1/10 their volume in freshly prepared 1-100 heparin in distilled water. Exudates containing a small number of red cells may be used in these tests. The exudates from both pleural cavities were combined, mixed, and distributed in 5 cc quantities in test tubes in which they were centrifuged slowly (about 10 minutes) until the supernatant suspensions were almost clear. The leucocytic sediments were combined and suspended in broth at pH 7.2 so that each cu mm contained 20,000 leucocytes. The leucocytes were kept warm during the preparation of the suspensions and subsequent tests.

The agglutinative test as finally developed was carried out as follows: 0.2 cc of toxin in various dilutions and 0.2 cc of control broth were pipetted separately into a series of tubes 10 x 75 mm. The freshly prepared leucocytic suspension was then pipetted into each tube in 0.2 cc quantities. The tubes were shaken immediately and placed in a water-bath at 37°C for 1½ hours, during which time each rack was taken out and shaken at intervals of 15 minutes. Following this the tubes were placed in the icebox at 4°C for 30 minutes and then read for leuco-agglutinin.

Results. Our results were always consistent when care was taken to prepare the leucocytic suspension exactly as described above.

Agglutination first appears in 1 or 2 of the tubes after approximately 50 minutes of incubation. The time of first appearance of agglutination is constant for any one filtrate. After further incubation the zone of agglutination spreads gradually into the tubes con-

[2] McClean, Douglas, *J. Path. and Bact.*, 1937, **44**, 47.

TABLE I.
Agglutination of Leucocytes by Staphylococcal Toxin.

	Strain of organism	Dilution of toxins undil.	1-2	1-5	1-10	1-20	1-50	1-100	1-200	1-400	Control broth
A	Wood	0	0	0	+	+++	++++	++++	++	+	0
B	103	++++	++++	++	+	0	0	0	—	—	0

+ to ++++ indicates varying degrees of agglutination.
++++ indicates complete agglutination.
0 indicates no agglutination.

taining diminishing amounts of toxin and, in the case of strong toxins, into the tubes containing increasing amounts of toxin. After 1½ hours' incubation, the leucocytes are clumped usually in one large gelatinous clot in the one or two tubes in which agglutination first appeared, leaving the surrounding fluid absolutely clear. The agglutinated clots are not broken up by shaking. A prozone always occurs unless the toxin is weak in agglutinative action.

In Table I are given the titrations of 2 toxins for leuco-agglutinins, A, a strong toxin showing the prozone effect and B, a weak toxin showing no prozone.

For purposes of calculation 1 unit of leuco-agglutinin represents the minimal amount of toxin that causes complete agglutination of leucocytes after 1½ hours at 37°C and 30 minutes in the icebox.

If films of the suspensions after incubation are prepared with Wright's stain, it is seen that the leucocytes in the prozonal tubes are completely disintegrated. In fact, when examined macroscopically, these tubes appear less opaque than the control tube of leucocytes and broth. This suggests that living intact leucocytes are necessary for agglutination to occur, which is borne out by the fact that for clear-cut results in these tests, it is important to use fresh leucocytic suspensions. Stains of smears made from the gelatinous clots recovered from the tubes with maximal agglutination show an amorphous blue-staining substance in which leucocytes can be made out only as faintly stained forms without demonstrable nuclei.

Correlation of leuco-agglutinins and leucocidins. Tests for leucocidins were carried out according to the "bioscopic" method of Neisser and Wechsberg with certain modifications. In order to compare exactly the leuco-agglutinin- and leucocidin-content of staphylococcal toxins, dilutions of toxins and leucocytic suspensions as described before were set up in duplicate. After 1 hour at 37°C both series of tubes were read for agglutination of leucocytes. Then one series of tubes was placed in the icebox and 0.05 cc of 1-10,000 methylene blue (Special Methylene Blue—LaMotte Chemical Co.)

and vaseline seals were added to each tube in the second series. The methylene blue tubes were then incubated overnight, this long incubation at 37°C being necessary in order to obtain complete reduction in the control tubes of leucocytes and broth. The medium used was always controlled for possible reduction by itself by incubating a tube of broth with methylene blue along with the other tests.

These tests showed a definite correlation between the amount of leucocidin and leuco-agglutinin in the toxins. There was regularly no reduction of methylene blue in the prozonal tubes nor in those showing complete agglutination, indicating that the leucocytes in these tubes had been killed. There was partial reduction usually in the tubes showing a ++ or +++ agglutination and complete reduction in the other tubes including the leucocyte and broth control. The control broth alone has never shown any reduction. Leuco-agglutinin and leucocidin also show a similar susceptibility to heat, both activities being destroyed when staphylococcal toxin is heated to 56°C for 1 hour. These results appear to indicate that staphylococcal leuco-agglutinin and leucocidin are closely related if not identical.

Agglutination of Rabbit Bone-marrow by Staphylococcal Toxin. Since, as has been demonstrated, staphylococcal toxin has an agglutinative effect on rabbit leucocytes, it seemed possible that it might also have a similar effect on rabbit bone-marrow. We found that such was the case. Exactly similar results in all respects were obtained when tests were run using bone-marrow suspension instead of leucocytic suspension and staphylococcal toxin.

In brief, the method of preparing bone-marrow suspensions is as follows: marrow obtained from the femur of a normal rabbit is quickly ground without sand in a warm mortar, and is taken up and emulsified in warm broth. Special care must be taken to keep the marrow warm throughout the entire process of preparing the suspension, otherwise a homogeneous suspension is not obtained and must be discarded. The suspension is pipetted with a warmed pipet into the tubes containing the toxin-dilutions which are standing in the water bath at 37°C.

Discussion and Summary. This report deals with an agglutinating reaction of staphylococcal toxin for rabbit leucocytes. When this test is carried out carefully, the results are clear-cut and remarkably consistent. Except for the fact that the leucocytic suspension is more exacting than a red cell suspension to prepare, the test is as easily and quickly set up and read as a test for bacterial hemolysin.

Our experiments demonstrate that there is a definite correlation between the content of leuco-agglutinin and the content of leucocidin

in any toxin, also that both activities are destroyed at the same temperature.

If we are correct in the interpretation of our results, leuco-agglutinin and leucocidin actually represent two stages of the action of the same toxic substance, and the leuco-agglutinin test, because of its simplicity and its clear-cut quantitative results, may be used to advantage over previous methods for the quantitative determination of leucocidin in staphylococcal toxins.

13569 P

Effect of Propazone on Respiration of Rat Tissue *in vitro*.*

FREDERICK A. FUHRMAN AND JOHN FIELD, 2ND.
From the Department of Physiology, Stanford University.

Propazone (5, 5-di-n-propyl-2, 4-oxazolidinedione) is one of a new series of compounds recently prepared and studied by Stoughton,[1] which has been shown to have hypnotic and anesthetic properties.[2] The structural relationship to the barbiturates and hydantoinates indicates that this agent might inhibit tissue respiration as do these other fixed hypnotics.[3] Propazone sodium† offers certain advantages in this type of study in that it is more soluble in water than the barbiturates and is nearly neutral in reaction. The effect of propazone on the respiration of rat liver, kidney cortex and cerebral cortex slices is described in the present paper. Twenty-four adult male rats were used.

The rate of oxygen consumption was measured by the Warburg manometric method, and is expressed in μl, N.P.T., per mg wet weight per hour (Q_{O_2}). The methods of preparation of tissue slices have been described previously.[4] The suspension medium was Ringer's phosphate, pH 7.35, containing 0.2% glucose. Propazone sodium was dissolved in glucose-free Ringer's phosphate and added

* Supported by grants from the Markle Foundation and the Fluid Research Fund of the Stanford University School of Medicine.

[1] Stoughton, R. W., *J. Am. Chem. Soc.*, 1941, **63**, 2376.

[2] Stoughton, R. W., and Baxter, J. H., *J. Pharm. and Exp. Ther.*, 1941, **73**, 45.

[3] Quastel, J. H., *Physiol. Revs.*, 1939, **19**, 135.

† We wish to thank Dr. R. W. Stoughton of the Mallinckrodt Chemical Works for his kindness in supplying propazone sodium.

[4] Fuhrman, F. A., and Field, J., 2d, *J. Pharm. and Exp. Ther.*, in press.

from the side-arms of the experimental vessels after 40 minutes. The Q_{O_2} during this period in which no propazone was present was used as the control in the calculation of inhibition. This is called Q_{O_2} C. The Q_{O_2} in the presence of propazone, Q_{O_2} P, was calculated for the period 45 to 75 minutes after addition of the drug, at which time the rate of oxygen consumption was constant. The intensity of inhibition is expressed by the ratio

$$\frac{Q_{O_2}\ P}{Q_{O_2}\ C}$$

which is termed the inhibition ratio.

The respiration of kidney cortex, liver and cerebral cortex slices was inhibited by propazone sodium in concentrations ranging from 50 to 500 mg % (2.4×10^{-3} to 2.4×10^{-2} M). This is shown in Table I. Q_{O_2} C and inhibition ratios are mean values obtained from 4 animals each.

If succinate or p-phenylenediamine were substituted for glucose as substrate, the inhibition produced by propazone was much less. With these substrates (0.02 M and 0.01 M respectively) the inhibition ratios for cerebral cortex with 250 mg % propazone were 0.94 and 0.96 respectively. Thus, as with other fixed hypnotics,[3] concentrations which produce considerable inhibition of respiration in a glucose medium have little or no effect in media containing succinate or p-phenylenediamine.

It is shown in Table II that the inhibition of respiration produced by propazone is reversible, at least in the case of kidney cortex. To obtain these data, Q_{O_2} C was determined for kidney slices, the drug then added, and Q_{O_2} P determined as before. After 40 minutes of inhibition, the slices were removed from the respirometer vessels, washed twice in Ringer's, placed in fresh suspension medium and Q_{O_2} measured again. Control slices receiving no propazone were treated similarly. Under these conditions, inhibition by 500 mg % propazone proved to be about 70% reversible.

TABLE I.
Effect of Propazone Sodium on Oxygen Consumption of Rat Tissues *in vitro*.
Medium: Ringer's-phosphate-glucose, pH 7.4, Temp. 37.5°C.

Tissue	Mean Q_{O_2} of controls	Inhibition ratios* Concentration Propazone Sodium, mg%			
		50	100	250	500
Liver	1.54	.78	.64	.47	.29
Kidney Cortex	4.48	.80	.71	.60	.26
Cerebral Cortex	2.26	.90	.78	.27	.15

*Inhibition ratio: Q_{O_2} Propazone/Q_{O_2} Control.

TABLE II.
Reversibility of Propazone Inhibition of Oxygen Consumption in Rat Kidney Cortex Slices.
Medium: Ringer's-phosphate-glucose, pH 7.4, Temp. 37.5°C.

	Propazone sodium, 500 mg%	Control
Mean Q_{O_2} before addition of propazone	5.30	4.60
Mean Q_{O_2} 35 min. after addition of propazone	1.80	4.60
Mean Q_{O_2} 45 min. after washing slices	3.20	3.95

Conclusions. Propazone sodium inhibits the oxygen consumption of rat kidney cortex, liver and cerebral cortex slices *in vitro*. For a given concentration of propazone, this inhibition is much more marked in media containing glucose than in those containing succinate or p-phenylenediamine. In kidney cortex the inhibition is about 70% reversible.

13570

Drug Prophylaxis Against Lethal Effects of Severe Anoxia. II. Alcohol, Amytal and Pentobarbital.

G. A. EMERSON, E. J. VAN LIERE AND JAMES L. MORRISON.

From the Departments of Pharmacology and Physiology, West Virginia University School of Medicine, Morgantown.

Effects of certain respiratory stimulants and other convulsants as antagonists to the lethal effects of acute anoxia have been reported.[1] Comparison of prophylactic effects of other types of agents through a standardized technic[2] is desirable, since many of the compounds previously reviewed[1] exhibit their protective action only under special conditions. Inasmuch as none of the convulsants affords complete prophylaxis against lethal effects of acute anoxia, it is of interest to observe the influence of various representative narcotics.

It was postulated[1] that, among others, an agent inhibiting the tendency toward convulsions in acute anoxia and an agent easily utilized as a nutrient under anoxic conditions might be of value if such agents did not unduly depress the nervous mechanisms governing respiration. Proper doses of ethyl alcohol may combine these 2 characteristics without too great respiratory depression. Accord-

[1] Emerson, G. A., and Van Liere, E. J., *Arch. internat. Pharmacodyn.*, 1940, **64**, 239.

[2] Emerson, G. A., Van Liere, E. J., and Morrison, J. L., to be published.

ingly, different doses of ethanol were administered to young adult white mice and tranquillizing and narcotic doses of a moderately long-acting and of a moderately short-acting barbiturate were administered to other mice, before exposure to anoxic anoxia. The barbiturates do not act as nutrients and thus serve as controls for the simple tranquillizing and narcotic effects of ethanol.

All agents were administered intraperitoneally. A 10% solution of absolute ethanol in physiologic saline, by volume, was injected as noted in Table I, and 60 cc/kg of saline were injected in other mice to control the effects of hydration. It was previously shown that treatment with 50 cc/kg of saline does not significantly alter the susceptibility of mice to lethal effects of anoxia.[1] Na amytal and Na pentobarbital were injected with 50 cc/kg of saline, and 50 cc/kg of saline were injected into control mice subjected simultaneously to anoxia.

Anoxic anoxia was produced in a decompression chamber resembling that described by Kolls and Loevenhart.[3] Since previous experience[1] demonstrated that the lethal range of anoxia is variable for mice on different days, each experiment was controlled by inclusion of a group of mice treated with saline, with simultaneous exposure to anoxia of these control mice and others receiving drugs. Anoxia was rapidly induced by reducing the tank pressure to a level simulating an altitude of about 10,000 ft. The mice were then maintained at this degree of anoxia for 10 minutes. It is felt that this treatment obviates any hazard of the occurrence of aeroembolism later.[2] The pressure in the tank was then reduced at a rate simu-

TABLE I.
Influence of Alcohol and Barbiturates on Resistance to Lethal Effects of Anoxia.

Agent	Dose	Time before exposure to anoxia, hours	Mortality ratio	Mean body wt of mice, g	
				Surviving	Dying
Ethanol	6.0 cc/kg	1	12/70	15.7	17.1
Saline	60. "	1	32/70	14.6	14.8
Ethanol	6.0 "	1	6/30	15.9	17.7
"	6.0 "	14	14/30	16.3	18.4
"	3.0 "	1	16/30	15.9	17.1
Saline	60. "	1	16/30	17.7	18.3
Na amytal	40. mg/kg	1	3/20	—	—
" "	80. "	1	7/20	—	—
" pentobarbital	25. "	1	6/20	—	—
" "	50. "	1	1/20	—	—
Saline	50. cc/kg	1	7/20	—	—

[3] Kolls, A. C., and Loevenhart, A. S., *Am. J. Physiol.*, 1915, **39**, 67.

lating an increase in altitude of about 1000 ft/min., until a simulated altitude of about 38,000 ft. was attained. At this pressure of 155 mm Hg, with a pO_2 of 31 mm, approximately half of the control mice succumbed in each instance. With corrections of readings of an open Hg manometer for barometric pressure, this level of anoxia corresponds to a simulated altitude of about 40,000 ft., or an atmospheric pressure of 141 mm, or a partial pressure of O_2 of 28 mm.

Three such experiments were carried out, with the results noted in Table I. Seventy mice treated with 60 cc/kg of 10% ethanol and 70 treated with 60 cc/kg of saline were simultaneously exposed to anoxia. Four groups of 30 mice each were also subjected to anoxia simultaneously; one group was treated with 60 cc/kg of 10% ethanol 1 hour before exposure to anoxia, another was similarly treated 14 hours before exposure to anoxia, a third group was treated with 30 cc/kg of 10% ethanol and a fourth group was treated with 60 cc/kg of saline, both of the latter groups 1 hour prior to exposure to anoxia. Finally, 4 groups of 20 mice each which had received barbiturates as noted in Table I, in 50 cc/kg of saline, and a control group of 20 mice which received 50 cc/kg of saline alone, were simultaneously exposed to anoxia.

Examination of Table I indicates that premedication with 6 cc/kg of ethanol 1 hour before exposure to anoxia greatly reduces mortality, from 48 of 100 control mice to 18 of 100 mice treated with ethanol. This reduction in the lethal effect of acute anoxia is highly significant statistically, with a p much less than 0.005, according to the criteria of Loewenthal and Wilson.[4] However, the same dose of ethanol given 14 hours prior to exposure to anoxia does not significantly alter mortality, nor does half the dose when administered 1 hour before exposure to anoxia. Mice treated with the higher dose of ethanol are in coma for several hours, but recover to apparently normal activity within 14 hours. Mice treated with the lower dose of ethanol are merely ataxic and somewhat more quiet than control mice before and during the exposure to anoxia. No further deaths occurred within 48 hours after the mice were subjected to anoxia.

Of the results with mice treated with barbiturates, only those of the group treated with 50 mg/kg of Na pentobarbital exhibit a significant reduction in mortality. The p for the significance of this reduction is exactly 0.05.

Also included in Table I are the mean body weights of the larger groups of mice surviving and succumbing under identical conditions

[4] Loewenthal, L. J. A., and Wilson, W. A., *Brit. Med. J.*, 1939, **2**, 110.

of treatment. These data tend to the conclusion that lighter mice are more resistant to lethal effects of anoxia than are heavier mice, but none of the differences in mean body weight is significant. The problem of correlation of body weight with susceptibility to anoxia is considered in more detail elsewhere.[2]

Comparison of the prophylactic effects of ethanol and the barbiturates indicates that the superiority of ethanol in this regard is dependent upon factors other than simple tranquillization of narcosis. Utilization of ethanol as a nutrient by anoxic tissues may be an important factor, but other differences in pharmacological action of the 2 types of narcotics must be sought for in larger anoxic mammals before definite conclusions may be drawn. Observations of the resistance to lethal effects of anoxia in mice treated with more selective depressants are in progress.

Within the range of doses used, it is improbable that ethanol or the barbiturates cause any appreciable histotoxic anoxia, for there is a complete lack of synergism of these agents with anoxic anoxia. These narcotics may cause a true histotoxic effect when applied to isolated tissues in high concentrations but there is no indication that a significant degree of histotoxic anoxia is produced by the sublethal amounts administered in the present study.

Since these studies are concerned with lethal effects of severe anoxic anoxia and only full narcotic doses of ethanol were found to be protective, no immediate applications to therapy in man are possible. The present results should not be construed to apply to milder degrees of anoxia, particularly as encountered in aviation practice, where alcohol is contra-indicated.[5, 6]

Summary. Full narcotic doses of ethyl alcohol significantly reduce the lethal effects of severe acute anoxic anoxia in mice if administered 1 hour prior to exposure to anoxia but not if administered 14 hours before exposure. Amytal and pentobarbital do not produce comparable prophylactic effects.

[5] Armstrong, H. G., *Principles and Practice of Aviation Medicine*, Baltimore, 1939.

[6] Barcroft, J., *Lancet*, 1920, **2**, 485.

13571 P

Biological Synthesis of Acetylcholine.

H. C. Chang, L. Y. Lee, C. W. Meng and Y. K. Wang.

From the Department of Physiology, Peiping Union Medical College, Peiping.

It was demonstrated that on incubation of the human placenta at a favorable temperature, *viz.*, 37-38°C, there is an increase of acetylcholine (AC) reaching a maximum at the 4th hour.[1] In view of the fact that no such increase occurs during incubation, if the tissue has been soaked in alcohol, or if the eserine (Es) saline extract of the tissue is incubated, the presence of an intracellular factor ("acetylizing endoenzyme") was postulated.[2]

It was later claimed that the action of the cholinesterase may be reverted by Es or body temperature, for the washed tissue which is almost free of the cholinesterase does not show the AC synthesis as the unwashed tissue does.[3] Sodium fluoride, quinine, prostigmine and berberine were found to have a similar action as Es.[4]

The suggestion of the intracellular factor was soon reinforced because certain forms of physical injury, such as grinding, freezing, and air-drying, applied to the placental tissue, destroy the intracellular factor, leaving the cholinesterase intact. Such tissue does not show AC-synthesis when incubated at 37.5°C or when Es is added. Similarly, cyanide kills the intracellular factor but spares the cholinesterase, and this cyanide-treated tissue fails to produce more AC in response to Es or the incubation at 37.5°C.[5]

Now it is possible to show that both factors appear to act together for the biological synthesis of AC in the presence of Es.

It is known that the pressed juice of the placenta contains the cholinesterase but not the intracellular factor, while the placental tissue which has been thoroughly washed by an intraarterial saline infusion contains chiefly the intracellular factor with only a trace of the cholinesterase.[5]

As shown in Table I, which gives 2 typical experiments of a series, the AC of the juice or the washed tissue is not changed by Es. But

---

1 Chang, H. C., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1001.

2 Chang, H. C., *Proc. 8th Annual Meeting of the Chinese Physiol. Soc., Peiping*, 1935, 7.

3 Chang, H. C., Lee, L. Y., and Meng, C. W., *Chinese J. Physiol.*, 1940, **15**, 343.

4 Chang, H. C., Lee, L. Y., and Meng, C. W., *Chinese J. Physiol.*, 1941, in press.

5 Chang, H. C., Lee, L. Y., Meng, C. W., and Wang, Y. K., *Chinese J. Physiol.*, 1941, in press.

TABLE I.
Action of Cholinesterase and an Intracellular Factor in Synthesis of AC.

Samples*	Total AC in γ† I	Total AC in γ† II	% destruction of added AC‡ by cholinesterase in 10 min. I	% destruction of added AC‡ by cholinesterase in 10 min. II
5 g washed tissue	216.0	152.0	5	15
5 g washed tissue + 150 γ Es	262.0	166.5	0	0
5 cc juice	1.7	1.6	82+	55
5 cc juice + 150 γ Es	1.7	1.7	0	0
5 g washed tissue + 5 cc juice	185.0	120.6	—	38
5 g washed tissue + 5 cc juice + 150 γ Es	559.8	361.7	—	0

*Three hours are allowed for the action to proceed in all the samples before the extraction.

†Assayed on the rectus as AC-chloride according to Chang and Gaddum.[6]

‡Assayed on the rectus according to Meng.[7]

when the juice is added to the washed tissue in the presence of Es, the AC-yield is very much increased, indicating clearly the collaboration of the two factors in the biological synthesis of AC.

Further experiments are in progress to determine the nature of the intracellular factor and to search for the source of the substrate concerned in this synthesis.

13572

Recovery of Antibody from Immune Precipitate of Type B Friedländer Bacillus.

SZU-CHIH LIU AND HSIEN WU.

From the Department of Biochemistry, Peiping Union Medical College, Peking, China.

In previous reports[1] from this laboratory, it has been shown that free antibody can be recovered from the immune precipitate or agglutinate of Type I pneumococcus by treating with dilute acid which causes a shift of the antigen-antibody equilibrium. The present communication extends our observation to the immune precipitate of another organism, Type B Friedländer bacillus.

[6] Chang, H. C., and Gaddum, J. H., *J. Physiol.*, 1933, **79**, 255.

[7] Meng, C. W., *Chinese J. Physiol.*, 1940, **15**, 143.

[1] Liu, S. C., and Wu, H., *Chinese J. Physiol.*, 1938, **13**, 449; *ibid.*, 1940, **15**, 465; PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 144; *ibid.*, 1940, **43**, 747; Lee, K. H., and Wu, H., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 65.

Antisera of horse and rabbit origin were prepared by immunizing animals with heat-killed Type B Friedländer bacilli. The specific polysaccharide of this organism was prepared according to the method of Heidelberger, Goebel, and Avery.[2] Polysaccharide was added to the antisera to form the specific precipitate. The precipitate was washed and suspended in 2% or 3% NaCl solution. The use of NaCl increases the percentage of recovery by decreasing the solubility of the precipitate.[3] Four cc portions of the suspension, containing about 5 mg N, were treated with equal volumes of dilute HCl of different concentrations according to the procedure previously described.[3] The percentage of recovery and the solubility of "acid-precipitates" (that is, the precipitate from which a part of the antibody has been set free by acid) at different pH's were calculated. Results are shown in Fig. 1. For the sake of comparison the curves

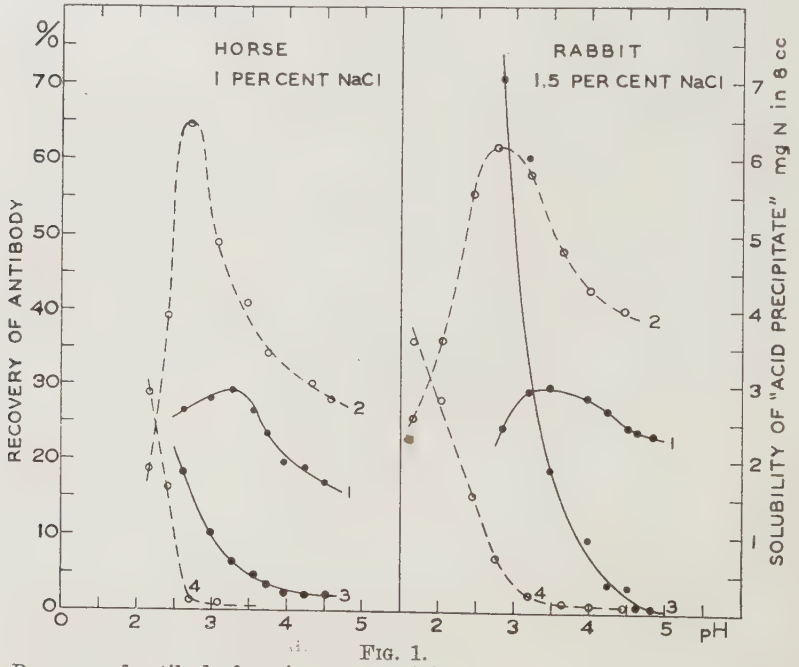


FIG. 1.

Recovery of antibody from immune precipitates of Type B Friedländer Bacillus at different pH's. t = 25°C.

1. Percent recovery from Friedländer Bacillus immune precipitate.
2. Percent recovery from Pn I immune precipitate.
3. Solubility of Friedländer Bacillus acid precipitate.
4. Solubility of Pn I acid precipitate.

[2] Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, **42**, 709.

[3] Liu, S. C., and Wu, H., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 144.

for Type I pneumococcus previously reported[3] are also shown in the figure.

For the precipitate obtained with horse serum in 1% NaCl, the optimal pH for recovery was 3.25, while for that with rabbit serum in 1.5% NaCl, the optimal pH was 3.50. These pH values are distinctly different from those (2.75 and 2.70) found for Type I pneumococcus under similar conditions. The optimal pH for the recovery of the antibody thus depends more on the nature of the specific polysaccharide than on the species of animal that produces the antibody.

The solubility of the acid-precipitate from rabbit serum was higher than that from horse serum, even though the solubility of former had been reduced considerably by the use of a higher concentration of NaCl (1.5%) than that (1%) used in the latter.

The maximal recovery was about 30% in both cases. This recovery is much lower than that (60%) previously reported for Type I pneumococcus. This difference is due to the higher solubility of the acid-precipitate of Type B Friedländer bacillus. The dissolved acid-precipitate decreases the percentage of recovery by recombination with the liberated antibody.

The purity of the recovered products was tested by quantitative precipitation. To 4 cc portions of the recovered antibody-solution (0.25 mg N per cc) were added 1 cc portions of the specific polysaccharide solution of different concentrations. The precipitates were washed and their N contents determined. At the point of maximal precipitation, the products recovered from the immune precipitates of horse and rabbit origin were respectively 89% and 85% precipitable.

13573

Ocular Absorption of Sulfonamide Derivatives After Local Application.*

SHIH-YI P'AN. (Introduced by Hamilton H. Anderson.)

From the Department of Pharmacology, Peiping Union Medical College, Peking, China.

It has been reported previously[1, 2] that sulfanilamide and sulfapyridine after local application to the eyes of experimental animals are absorbed in greater amounts by ocular tissues and fluids than occurs when these agents are administered orally.[3–7] The ocular absorption of sulfathiazole has recently been studied by Bellows and Chinn.[8] Dogs given 0.2 g per kg of the drug, which was dissolved in HCl before administration, after 4 hours developed blood concentrations of 7.4 mg %. The amounts of sulfathiazole found in ocular tissues and fluids were: sclera 10.4, conjunctiva 8.4, chorioretinal layers 4.9, cornea 2.0, vitreous humor 0.9, aqueous humor 0.4, and lens 0 mg %. When an aqueous suspension of a similar dose of sulfathiazole was given orally the concentrations were considerably lower: blood 1.7, conjunctiva 2.9, chorioretinal layers 1.7, aqueous humor 0.3, vitreous humor 0.2 and lens and cornea 0 mg %. Similar results have been reported on cats by Scheie and Souders,[9] who found that a very small amount of sulfathiazole diffuses into the aqueous humor as compared with the blood concentration. In addition they have observed that sulfadiazine, like sulfanilamide, penetrates into the aqueous humor when given orally. Turkell and Wilhelm[10] have shown that

* Sulfaguanidine and sulfadiazine were supplied by the Lederle Laboratories, Pearl River, N.Y.; N^4-nicotinylsulfanilamide by the Lilly Laboratories, Indianapolis, Ind.; N^1-nicotinylsulfanilamide was prepared by Dr. Chas. H. Ch'eng of our laboratory, and N^1,N^4-dinicotinylsulfanilamide by Dr. Troy Daniels, University of California, College of Pharmacy, San Francisco, Calif.

1 P'an, S. Y., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 31.

2 P'an, S. Y., *J. Pharm. and Exp. Therap.*, 1941, **72**, 31.

3 Rumbo, V. C., *Am. J. Ophth.*, 1938, **21**, 739.

4 Bellows, J. G., and Chinn, H., *J. A. M. A.*, 1939, **112**, 2023.

5 Mengel, W. G., *Arch. Ophth.*, 1939, **22**, 406.

6 Luo, T. H., and P'an, S. Y., *Chin. M. J.*, 1940, **58**, 167.

7 Meyer, K., Bloch, H. S., and Chamberlain, W. P., Jr., *Am. J. Ophth.*, 1941, **24**, 60.

8 Bellows, J. G., and Chinn, H., *Arch. Ophth.*, 1941, **25**, 294.

9 Scheie, H. G., and Souders, B. F., *Arch. Ophth.*, 1941, **25**, 1025.

10 Turkell, J., and Wilhelm, S. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 97.

sulfathiazole was secreted in tears in amounts far less than in the blood after oral use.

Since sulfanilamide and related agents have been used in the therapy of ocular diseases it was believed desirable to compare the absorptive rates of 8 sulfonamide derivatives after local application to the eyes of rabbits. The technic employed has been described elsewhere.[1] For N^4-nicotinylsulfanilamide and N^1,N^4-dinicotinylsulfanilamide a method not previously described has been used.†

Table I summarizes the results of the present series of experiments together with those obtained with sulfanilamide and sulfapyridine as previously reported.[1, 2] The N^4-nicotinylsulfanilamide was not included in the table because none of it was found in any of the ocular tissues or fluids or blood after its local application to the eyes of 6 rabbits. It may be seen that the absorption of N^1-nicotinylsulfanilamide and N^1,N^4-dinicotinylsulfanilamide was comparable to that of sulfanilamide and sulfapyridine. The concentrations of sulfathiazole, sulfaguanidine and sulfadiazine in the cornea of rabbits locally treated with each drug were definitely higher, and of the conjunctiva were equal or above those found in these tissues after oral administration of reasonable doses of the 3 derivatives. Local application of these 3 agents resulted in ineffective concentrations developing in the lens, sclera, chorioretinal layers, aqueous humor and vitreous humor as well as in the blood. No evidence of chemosis or other signs of irritation to ocular tissues were noted after the application of any one of these derivatives. Six or more eyes were treated with each sulfonamide derivative reported in this experiment.

To be of value in the therapy of localized ocular infections, one would assume that therapeutically effective tissue concentrations are needed. Local application of powdered sulfonamides to the normal rabbit's eye for 60 minutes resulted in the accumulation of significant amounts of all agents in the conjunctiva and cornea, except after

† Sodium hydroxide instead of HCl was used for hydrolysis at the suggestion of Dr. Jack Finnegan, University of California Medical School, San Francisco. Ocular tissues were mixed with an appropriate amount of 85% alcohol so that each cc contained 20 mg of tissue. The mixture was filtered and 2 cc of the filtrate was pipetted into 17 × 150 mm test tubes graduated at 10 cc. One cc of 2N NaOH and 3 cc of water were added and the tube was placed in a bath of boiling water for 2½ hours. The mixture was neutralized to phenolphthalein with N HCl and diluted with distilled water to 10 cc. After cooling, 2 cc of 0.025% $NaNO_2$ was added and 3 minutes later 5 cc of 0.4% dimethyl-α-naphthylamine in 95% alcohol was added and the solution was compared with an appropriate standard in a Klett-Summerson photoelectric colorimeter using filter No. 54. For blood, 0.1 cc was added to 5 cc of 85% alcohol and the mixture was filtered and 2 cc of the filtrate was treated in a similar manner.

TABLE I.
Amounts of Sulfonamide Derivatives Found After Local Application (in mg per 100 g or cc).

(R_1)-N-S(=O)(=O)-C6H4-N-(R_4)	Conjunctiva	Cornea	Sclera	Lens	Chorio-retinal layers	Aqueous humor	Vitreous humor	Blood†
R_1=H R_4=H Sulfanilamide*	89.8±14.0	185.4±13.2	23.3±4.6	8.7±1.8	12.3±1.2	54.8±5.3	1.1±0.2	0
R_1=  R_4=H Sulfapyridine**	47.1±11.2	30.3±6.0	10.9±3.6	2.4±0.8	0	4.7±1.2	0	0
R_1=  R_4=H Sulfathiazole	8.9±1.7	23.5±1.9	3.2±0.5	0	0	2.1±0.3	0.1±0.1	0
R_1=  R_4=H Sulfaguanidine	4.9±1.0	11.5±2.5	0.9±0.1	0.4±0.2	0	0.8±0.1	0.2±0.2	0
R_1=  R_4=H Sulfadiazine	4.9±0.7	9.4±0.8	1.6±0.3	0	0	2.2±0.5	0	0
R_1=  R_4=H N^1-nicotinyl-sulfanilamide	10.7±2.9	140.5±15.6	13.0±3.1	4.6±0.9	9.2±1.8	37.7±10.5	0.5±0.2	0
R_1, R_4=  N^1,N^4-dinicotinyl-sulfanilamide	8.6±1.7	61.6±8.8	6.8±1.8	0.3±0.3	0.3±0.3	7.2±1.6	0	0

†Effective blood concentrations for systemic activity are: sulfanilamide 10 mg%, sulfapyridine, sulfathiazole, sulfaguanidine and sulfadiazine 5 mg%.

*See PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 31.

See *J. Pharm. and Exp. Therap.*, 1941, **72, 31.

N^4-nicotinylsulfanilamide. Sulfanilamide and N^1-nicotinylsulfanilamide are absorbed in effective amounts by all tissues and fluids except the vitreous humor. Sulfapyridine and N^1,N^4-dinicotinylsulfanilamide are found in therapeutic concentrations in the conjunctiva, cornea, sclera, and aqueous humor. The finding that sulfathiazole, sulfaguanidine and sulfadiazine were present in effective concentrations only in the conjunctiva and cornea indicates that they were slowly absorbed by other ocular tissues and fluids and possibly remained in the conjunctiva and cornea for a comparatively longer time. It may, therefore, point to preference for these 3 drugs when ocular infections are localized to the conjunctiva and cornea.

13574 P

Response of Sulfonamide-fast Pneumococci to Penicillin.

H. M. Powell and W. A. Jamieson.

From the Lilly Research Laboratories, Indianapolis, Indiana.

Schmidt and Sesler[1] have shown that hydroxyethylapocupreine is effective against both sulfonamide-fast and parent strains of pneumococci; however, the degree of effectiveness against parent strains was less and the toxicity was more than that of sulfapyridine in comparable chemotherapeutic dosage.

We have conducted similar comparative experiments with penicillin and sulfapyridine in mouse infections with Types I, II, and III pneumococci, a "fast" and parent strain of each type being used. The penicillin was prepared by Dr. J. M. McGuire and Mr. F. G. Jones of the Lilly Research Laboratories; this substance comprised a brown powder about 200 times as bacteriostatic for staphylococci as the culture filtrate from which it was derived. The six strains of pneumococci were obtained through the kindness of Dr. L. H. Schmidt of Christ Hospital, Cincinnati. Our experiments are described briefly as follows:

The stock pneumococcus cultures were passaged through mice once each week. Directly after passage, 16-hour rabbit blood broth subcultures were prepared. For therapy, a group of 240 mice of about 20 g weight were each injected with 10^{-4} cc (about 10,000 M.L.D.) of the various subcultures of pneumococci. Subgroups of 20 mice infected with each strain were treated respectively with penicillin and sulfapyridine, as shown in the upper 3 sections of Table I. One hundred forty-four additional nontreated control mice were injected in groups of 4 with doses of 10^{-4} to 10^{-9} cc of the various pneumococcus strains to determine the degree of virulence. All of the strains proved of high virulence at the time of use, as shown in the lower section of Table I.

Penicillin therapy comprised 10 oral doses of 20 mg each, 4 doses being given at 2-hour intervals on each of the first and second days and 2 doses being given with an 8-hour interval on the third day. A dose of 5 mg of sodium bicarbonate was given with each dose of penicillin as a protection against gastric acidity. Sulfapyridine therapy comprised 5 oral doses of 30 mg each, 2 doses being given with an 8-hour interval on each of the first and second days and one dose

1 Schmidt, L. H., and Sesler, C. L., *J. Phar. and Exp. Therap.*, 1941, **72**, 311.

TABLE I.
Comparative Response of Parent and Sulfapyridine-fast Pneumococci to Penicillin and Sulfapyridine.

Pneumococcus 10^{-4} cc			Number of mice dead on days indicated or survived 14 days (S)									Per cent survived	Mean length of life (days)
Type	Strain	Therapy	1	2	3	4	5	6	7	8-14	S		
I, McGovern	parent	1					1				19	95	13.5
	"	2			1			2	2	2	13	65	11.6
	fast	1								3	17	85	13.6
	"	2	8	12							0	0	1.6
II, C H	parent	1				1					19	95	13.5
	"	2			2	4	12	2			0	0	4.7
	fast	1								1	19	95	14.0
	"	2	2	13	4	1					0	0	2.2
III. C H A	parent	1	1	1	2	3					13	65	10.1
	"	2		5	5	5	5				0	0	3.5
	fast	1	1	2	3	3					11	55	9.0
	"	2	12	6	2						0	0	1.5

Virulence controls; all types and strains (cc)	Type I, McGovern		Type II, C H		Type III, C H A	
	Parent	Fast	Parent	Fast	Parent	Fast
10^{-4}	4/4*	4/4	4/4	4/4	4/4	4/4
10^{-5}	4/4	4/4	4/4	4/4	4/4	4/4
10^{-6}	4/4	4/4	4/4	4/4	4/4	4/4
10^{-7}	4/4	4/4	4/4	4/4	4/4	4/4
10^{-8}	4/4	3/4	3/4	3/4	2/4	3/4
10^{-9}	0/4	1/4	0/4	0/4	1/4	3/4

Therapy: 1 = penicillin 20 mg × 10 doses.
2 = sulfapyridine 30 mg × 5 doses.
*Mice dead/mice used.

being given on the third day. Initial drug doses in both instances were given within an hour after infection, and all overnight intervals when no drug was given were 16 hours. Details of pneumococcus mouse infections of this order and effective sulfonamide chemotherapy have already been described.[2, 3]

It is observed from the results in Table I that sulfapyridine has a decreasing order of antipneumococcal effectiveness against Type I parent strain, Type II parent strain, Type III parent strain, and Types I, II, and III fast strains; the degree of effectiveness against these 3 latter strains in fact is practically *nil*. Penicillin, in contrast, appears highly effective against both parent and fast strains of Types I and II and moderately effective against both parent and fast strains of Type III pneumococci.

[2] Powell, H. M., and Chen, K. K., *J. Ind. State Med. Assn.*, 1940, **33**, 503.
[3] Powell, H. M., and Chen, K. K., *J. Ind. State Med. Assn.*, 1941, **34**, 602.

Further experiments with *Streptococcus hemolyticus* and *Staphylococcus aureus* will be reported in the near future.

The penicillin employed has now been concentrated by chemical means, thus permitting the oral administration of substantially smaller doses than those reported here.

We desire to express to Dr. G. H. A. Clowes our indebtedness for advice and suggestions in the conduct of these experiments.

Conclusions. Penicillin is an effective chemotherapeutic agent against both parent and sulfonamide-fast pneumococci in mouse infection experiments.

13575

Formation of Angiotonin-like Pressor Substance from Action of Crystalline Pepsin on Renin-Activator.

O. M. HELMER AND IRVINE H. PAGE.

From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis, Indiana.

The interesting communication of Croxatto and Croxatto[1] in which they describe the formation of a pressor substance similar in properties to angiotonin or hypertensine prompted us to repeat their work. We wished to ascertain whether the same results could be obtained by using crystalline pepsin instead of the commercial product. Further, the effect of pH on this reaction as well as on that between renin and renin-activator was studied.

Methods. Preparation of the Renin-activator. For the preparation of renin-activator ox serum[2, 3] was used. One volume of 3 M potassium phosphate at pH 6.5 was added to an equal volume of ox serum and the resulting precipitate discarded. To each liter of the clear filtrate 500 cc of 3 M phosphate was added to raise the phosphate concentration to 2 molar. This precipitate which contains the active material was collected on Buchner funnels.

The precipitate was dissolved in distilled water and dialyzed against running tap water. The precipitate which forms in the sac was discarded and the residual solution reprecipitated between 1.5 and 2

1 Croxatto, H., and Croxatto, R., *Science*, 1942, **95**, 101.

2 Kohlstaedt, K. G., Helmer, O. M., and Page, I. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **39**, 214.

3 Kohlstaedt, K. G., Page, I. H., and Helmer, O. M., *Am. Heart J.*, 1940, **19**, 92.

molar potassium phosphate. The precipitate was suspended in as small a volume of distilled water as possible and dialyzed until free of potassium phosphate.

Any precipitate which formed on dialysis was removed by centrifuging, and the final volume adjusted so that 1 cc was equivalent to 10 cc of original ox serum. Sodium chloride was added to a concentration of 0.9%.

The renin-activator and enzyme solutions were adjusted to the desired pH with hydrochloric acid or sodium hydroxide, using a glass electrode as indicator. After mixing, the solutions were incubated for 10 minutes at 40°C, boiled for 10 minutes, cooled and centrifuged. The clear supernatant fluid was used for assay in pithed cats. The latter were prepared by pithing under pentobarbital anesthesia. The crystalline pepsin was dissolved in 85% glycerol and 1 cc ≡ 3.1 mg P.N. per cc. It was diluted 1:1 with saline. The renin was of such pressor activity that 0.2 cc produced a rise of ar-

TABLE I.
Formation of Pressor Substance from Interaction of Pepsin and Renin-Activator as Indicated by the Reaction of Pithed Cats.

Amt of activator, cc	pH		Exp. No.	Vol. inj. cc	Rise in pressure, mm Hg.
5	6.5	5 cc 1:50 dilution renin	2460	0.5	52
5	6.5	5 " saline		0.5	0
5	6.5	U.S.P. pepsin ≡ 10 mg		0.5	0
5	2.0	U.S.P. " ≡ 10 "		0.5	6
5	2.0	U.S.P. " ≡ 10 "		1.0	14
5	2.0	U.S.P. " ≡ 5 "		0.5	0
5	2.0	5 cc 1:50 dilution renin	2462	1.0	0
5	2.0	U.S.P. pepsin ≡ 100 mg		0.5	44
5	2.0	U.S.P. pepsin ≡ 50 mg	2466	0.5	12
5	2.0	U.S.P. " ≡ 50 "		1.0	19
5	6.5	U.S.P. " ≡ 50 "		0.5	0
5	6.5	U.S.P. " ≡ 50 "		1.0	6
5	7.0	5 cc 1:50 dilution renin		0.5	20
5	7.0	5 " 1:50 " "		1.0	38
5	4.0	5 " 1:50 " "		0.5	0
5	4.0	5 " 1:50 " "		1.0	0
5	2.0	5 " 1:50 " "		0.5	0
5	2.0	5 " 1:50 " "		1.0	0
3	2.0	3 cc crystalline pepsin	2469	1.0	10
3	2.0	U.S.P. pepsin ≡ 30 mg		1.0	12
3	6.5	3 cc 1:50 dilution renin		1.0	16
5	2.0	5 cc crystalline pepsin	2470	1.0	24
5	2.0	5 " U.S.P. pepsin ≡ 50 mg		1.0	30
5	6.5	5 " 1:50 dilution renin		1.0	34
		Saline 1 cc and crystalline pepsin content		1.0	0

terial pressure of 50 mm Hg in a cat under pentobarbital anesthesia.

Results. Our results entirely confirm those of Croxatto and Croxatto in demonstrating that incubation of renin-activator with commercial pepsin at pH 2.0 leads to the formation of a pressor substance with properties similar to those of angiotonin (Table I). The crystalline as well as the U.S.P. pepsin caused the formation of a heat stable substance able to produce a sharp rise in the arterial pressure of pithed cats.

The reaction between pepsin and renin-activator proceeds at a satisfactory rate at pH 2.0 but at pH 6.5 it is practically stopped. In sharp contrast to the effect of pH on the pepsin reaction is its action on that between renin and renin-activator. At pH 2.0 to 4.0 apparently no angiotonin is formed while at pH 6.5 to 7.0 it is formed abundantly.

It is of interest to note the relatively much greater potency of renin in the formation of angiotonin as compared with crystalline pepsin. The renin which we employed was free of the enzyme contained in kidney extracts which destroys angiotonin.

Dr. John Northrup was kind enough to supply us with some crystalline pepsin for our study. We are very grateful to him.

Summary. We have been able to confirm the observations of Croxatto and Croxatto that commercial pepsin at pH 2.0 reacts with renin-activator to form a substance similar to and probably identical with angiotonin. Furthermore, we found crystalline pepsin active. The reaction proceeds satisfactorily at pH 2.0 but is halted at pH 6.5 in sharp contrast to that between renin and renin-activator. At pH 6.5 to 7.0 the latter reaction yields angiotonin abundantly but none is formed at pH 2.0 to 4.0.

13576

Effects of Some Respiratory Inhibitors on Respiration and Reconstitution in Tubularia.*

Florence Moog and Sol Spiegelman. (Introduced by L. G. Barth.)

From the Department of Zoology, Columbia University, and the Marine Biological Laboratory, Woods Hole, Mass.

Morgan[1] showed that the polarity of stem sections of Tubularia can be reversed if the distal end, which has the higher tendency to reconstitute, is thrust into sand. Since that time several other researches have similarly indicated that access to oxygen may be the determining factor in polarity and rate of reconstitution; and recently Barth[2] showed that the rate of reconstitution can be elevated by increasing the oxygen tension of the water, and depressed by decreasing the tension.

These results indicated the desirability of studying the respiratory mechanisms underlying reconstitution. Accordingly a series of correlated experiments were performed to determine the effects of potassium cyanide, which is believed to attack cytochrome oxidase, and phenyl- and ethyl-urethane, which probably interfere with dehydrogenases, on respiration and reconstitution in Tubularia. Sodium azide was also used, although the nature of its effect at the pH of sea water is not known.

Methods. The solutions were made up fresh each week in filtered sea water, and when necessary adjusted to pH 8.2 with hydrochloric acid. Six mm sections cut from the most distal portion of freshly gathered stems, uniform in translucence, length, and diameter, were used. Most of the reported experiments were performed in July and August on Tubularia collected from the cold waters of Cape Cod Bay.

In the reconstitution experiments the stems were kept in partly filled, tightly stoppered flasks, which were shaken at intervals to redistribute oxygen. The stems were kept in flasks until they reconstituted, or otherwise for four or five days. They were counted as totally inhibited, with rate of reconstitution zero, if after being removed to fresh sea water they developed hydranths. The rate was

* Aided by a grant from a Rockefeller Foundation fund administered by H. B. Steinbach.

1 Morgan, T. H., *Arch. Entw.*, 1903, **16**, 125.

2 Barth, L. G., *Physiol. Zool.*, 1938, **11**, 179; *Biol. Bull.*, 1940, **78**, 366.

determined according to the method formulated by Barth,[3] as length of the unemerged hydranth divided by the time to its constriction.

The respiratory measurements were made with Warburg manometers, 20 stems being used in 2 cc of fluid. Measurements were carried out over a period of approximately 24 hours, the time required for the primordium to be fully formed in sea water at the temperature used, 19°. For every sample in the Warburgs a parallel control was kept in standing solution for the accurate measurement of reconstitution rate. Although shaking caused the stems in the manometers to reconstitute at a slightly higher rate than the standing controls, it did not change the relative effects of the various inhibitors.

Results. Table I summarizes the results of a number of representative experiments involving all the agents used. The table shows the tendency of all values to vary with the batch of stems and with the season.

TABLE I.

	Reconstitution rate				Q_{O_2} (mm^3 O_2/stem/hour)			
Agent	Exp. No.	Control	Experimental	% inhibition	Exp. No.	Control	Experimental	% inhibition
Potassium cyanide								
5×10^{-5} M	9	22.4	11.4	49.1	9	1.28	0.40	68.8
1×10^{-4} M	9	22.4	7.0	68.8	9	1.28	0.19	85.3
1×10^{-4} M	10	33.6	8.5	74.6	10	1.54	0.48	68.8
1×10^{-3} M	4	25.6	0	100	4	2.64	0.44	83.5
KCN + 0.002% methylene blue								
5×10^{-5} M + MB	9	22.4	10.5	53.1	9	1.28	0.08	93.8
1×10^{-4} M + MB	9	22.4	6.7	70.1	9	1.28	0.16	87.5
1×10^{-4} M + MB	10	33.6	10.5	68.8	10	1.54	0.40	74.0
Sodium azide								
5×10^{-5} M	12	15.7	13.8	12.1	12	2.24	1.98	11.7
5×10^{-4} M	12	15.7	15.4	1.8	12	2.24	1.92	14.3
1×10^{-3} M	12	15.7	6.3	60.2	12	2.24	2.04	8.8
2×10^{-3} M*	7	38.0	5.0	86.9	3	2.67	2.04	23.5
Phenyl urethane								
2×10^{-5} M	9	22.4	25.0	(11.3)†	9	1.28	1.24	3.0
1×10^{-4} M	9	22.4	2.7	87.8	9	1.28	1.24	3.0
1×10^{-4} M	10	33.6	4.9	85.4	10	1.54	1.39	9.5
Ethyl urethane								
5×10^{-2} M	11	21.8	0	100	11	1.20	0.92	23.4
5×10^{-2} M	9	22.4	0	100	9	1.28	1.36	(6.3)†
5×10^{-2} M	10	33.6	24.6	26.8	10	1.54	1.40	9.0

*Although these values are not from the same experiment, they were obtained from stems collected in the same locality within the same week.

†The figures in parentheses indicate a per cent increase above the rate of the control.

[3] Barth, L. G., *Biol. Bull.*, 1938, **74**, 155.

1. *Cyanide.* Potassium cyanide effects a decrease in the reconstitution rate in concentrations as low as 6×10^{-6} M, and completely prevents reconstitution in solutions of 2×10^{-4} M, or a little higher. The action of cyanide is reversible, however, and stems kept in 1×10^{-3} M for 48 hours reconstitute when transferred to sea water. Cyanide cuts the reconstitution rate principally by lengthening the time of formation of the new hydranth, for the length of the hydranth is decreased only in the strongest solutions; this observation holds true also for the 3 other inhibitors used.

On the respiration of reconstituting stems cyanide also exerts a powerful inhibitory effect, but it is not capable of eliminating respiration altogether; even in 1×10^{-3} M respiration was maintained at 16% of the control value. Evidently there is a considerable residual respiration in stems in which reconstitution is completely blocked.

2. *Methylene Blue.* Because methylene blue is sometimes able to accept hydrogen and so to serve in place of the cytochrome system, 0.002% solutions were tried alone and with cyanide. But in no case did methylene blue alleviate the effect of cyanide on either reconstitution or respiration; nor did it have any perceptible effect when used alone.

3. *Sodium Azide.* Azide begins to affect reconstitution in 5×10^{-6} M solution, and completely suppresses it in solutions of a little more than 1×10^{-3} M. As in the case of cyanide the effect is reversible, for stems kept in 3×10^{-3} M for 48 hours reconstitute on transfer to sea water. That the intimate effect of azide differs from that of cyanide, however, is shown by the fact that azide solutions as concentrated as 2×10^{-3} M, which were found invariably to cut the reconstitution rate by at least 80%, alter the rate of oxygen uptake only moderately or not at all.

4. *Phenyl Urethane.* This narcotic inhibits reconstitution in concentration from about 5×10^{-5} M to 1.3×10^{-4} M. In this range inhibition is reversible, for weaker stems which failed to reconstitute in the urethane solutions do so when placed in fresh sea water, but in slightly higher concentrations the stems are rapidly killed. Below 5×10^{-5} M there is a decided acceleration of the reconstitution rate, which in one case rose to 135% of the control value. But in the respiration experiments, as the table shows, neither high nor low concentrations exerted any marked effect on the oxygen consumption.

5. *Ethyl Urethane.* Results obtained with ethyl urethane closely parallel those obtained with phenyl urethane. The inhibiting range is from 1×10^{-2} M to 6×10^{-2} M, and inhibition is reversible within this range. Concentrations a little above the upper limit consistently raised the reconstitution rate by not more than 10%, and solutions

slightly stronger than the lower limit were lethal. As can be seen from the table, however, a concentration which is capable of suppressing reconstitution completely does not exert a strongly inhibitive influence on respiration.

Discussion. The principal point established by these experiments is that only a very small part of the overall respiration is involved in the process of reconstitution. The urethanes and perhaps azide apparently act selectively on those dehydrogenases which belong to the system or systems directly associated with the metabolism for reconstitution.[4] Under these conditions only a small part of the respiration is affected, the remaining oxidative systems being unaffected or even accelerated. Cyanide, on the other hand, by attacking the ferrocatalyst in the main portion of the aerobic respiration, inhibits the corresponding oxidative systems equally and leaves only a low cyanide-insensitive respiration which is incapable of serving even the small respiratory needs of reconstitution. But this incapacity is not caused by quantitative insufficiency. Rather, the presence of cyanide blocks the flow of energy through many pathways, among which is that part of the respiratory cycle on which reconstitution depends. Barth's findings (1940, loc cit.) with varied oxygen tensions might be similarly interpreted on the basis of an unspecific stimulative or depressive effect on all oxygen-consuming systems.

This result is not surprising, for it has been found in numerous cases that an acitvity can be stopped without any marked effect on the respiration. If the oxidative system underlying the activity (in this case reconstitution) is only a small percentage of the total respiration, then neither stimulating nor suppressing that system would noticeably affect the overall respiration, although both events would be markedly reflected in the change in the rate at which the activity is carried on.

Summary. (1) Cyanide, azide, phenyl urethane, and ethyl urethane can slow or stop reconstitution in Tubularia; methylene blue does not alter the effect of cyanide. (2) Cyanide strongly inhibits oxygen consumption in the concentrations which affect reconstitution, but it does not suppress oxygen consumption entirely. (3) Azide and the urethanes do not have a marked effect on oxygen consumption in the concentrations in which they strongly decrease reconstitution. (4) These findings are compatible with the fact that oxygen tension influences the rate of both reconstitution and respiration.

[4] *Cf.* Fisher, K. C., *Biol. Bull.*, 1941, **80**, 282.

13577

Effect of Urea in Sulfonamide Base on Healing of Clean Skin Wounds in Rabbits.*

Magnus Olson, Ethel Slider, William G. Clark and Roger MacDonald.

From the Department of Zoology, University of Minnesota, Minneapolis, Minn.

Urea has been used in the treatment of infected wounds since 1915. Since that time some 40 papers on the subject have appeared, but only one completed report (Chiariello[1]) gives data on the effect of urea on relatively uninfected experimental wounds. A note claiming beneficial effects of urea in experimental wounds in animals appeared in 1940,[2] but no supporting data were included. An abstract by Greenbaum and Aye[3] mentions some experimental work on effects of allantoin, allantoin-dipiperazine, 40% urea, and saline on wound healing in the fowl. Allantoin is claimed to exert the most beneficial effect, and urea the least. No data are given. All other papers have usually been concerned with uncontrolled infected clinical cases.

Low concentrations of urea are claimed by Robinson[4] to stimulate proliferation in wounds, whereas Holder and MacKay,[5] and many others claim that rather than acting as a stimulant to proliferation, only in strong concentrations does urea exert its beneficial effects through its cleansing solvent action in necrotic wounds, because of its bactericidal qualities, and because it removes inhibitors of topically applied sulfonamides.

Chiariello,[1] produced open skin defects in guinea pigs and rabbits by cautery and surgery, and measured the time to complete healing of the defects treated with 4% urea as compared with dry controls, or controls treated with tannic acid, saline, or olive oil. No effects were noted other than a subjectively observed slight decrease of the latent periods of the wounds produced by cautery.

Rabbits were used in the present study. Circular defects, 1-3 cm in diameter, were incised through the corium to the panniculus carnosus.

* Supported by grants from the Graduate School and from the Medical Research Committee of the University of Minnesota. Assistance was furnished by the personnel of Works Projects Administration, Official Project No. 165-1-71-124, Subproject No. 383; and National Youth Administration, Project No. 169-120.

1 Chiariello, A. G., *Folia Med.*, 1939, **25**, 61.

2 Annotations, *Lancet*, 1940, **238**, 1132.

3 Greenbaum, F. R., and Aye, C. E., *101st Proc. Am. Chem. Soc.*, 1941.

4 Robinson, W., *Am. J. Surg.*, 1936, **33**, 192.

5 Holder, H. G., and MacKay, E. M., *Ann. Surg.*, 1939, **110**, 94.

No aseptic precautions were taken. The wounds were protected by flanged celluloid collars with removable caps. The flanges were made fast to the shaved skin with flexible collodion. The wounds were treated with a water-soluble jelly consisting of a 4% gum-karaya glyceride, 1% boric acid as a fungicide and 0.5% sulfanilamide or 5% sulfathiazole. Three different concentrations of urea, 40%, 10%, and 2% were incorporated in the jelly base. One control and one experimental defect were incised in each animal symmetrically on either side of the dorsal midline at the shoulders. The wounds were redressed and traced on transparent cellophane every 48 hours for 12-14 days. The animals were hobbled and collared to prevent access to the wound caps. Anesthesia (pentothal-sodium†) was employed at each dressing, to facilitate more accurate tracings.

Blood agar plates with a 24-hour *Staphylococcus aureus* culture showed a marked inhibitory effect of the jelly base containing 5% sulfathiazole, and no effect of one containing 0.5% sulfanilamide. 40% urea did not enhance the inhibition by sulfathiazole.‡ Sterile swabs taken from the surface of the urea-sulfathiazole treated wounds were cultured in Brewer's thioglycollate broth and on blood agar plates. Gamma type non-hemolytic streptococci were obtained,§ but that they developed only after transfer from the bacteriostatic sulfathiazole medium of the wounds was shown by their absence in gram-Weigert stained sections of the tissues.

At the end of 12-14 days, the animals were sacrificed and paraffin sections of Bouin or formalin fixed tissue from the center of the wounds were stained by gram-Weigert's, Mallory's, and hematoxylin-eosin methods. The gram-Weigert's stain permitted an estimate of the presence or extent of bacterial contamination. This technic revealed a few relatively heavy contaminations of gram-positive cocci and bacilli in the defects treated with 40% urea and 0.5% sulfanilamide, and these were largely restricted to the surface regions. Very low-grade or no infections were found in those treated with the lower concentrations of urea and 5% sulfathiazole.

The slides of the cross-sectioned tissue were photographed and the rate of increase in area of the granulation tissue was estimated by planimetric means. Measurements from photographs and from the first cellophane tracings (made 48 hours after operating) also permitted calculation of the rate of epithelization.

† The pentothal-sodium was supplied by the Abbott Laboratories.

‡ Acknowledgment is made to Dr. Ernst Strakosch, of the Department of Dermatology, University of Minnesota, for making these studies.

§ Acknowledgment is made to Mr. Charles H. Drake, of the Department of Bacteriology, University of Minnesota, for making these studies.

The results of our preliminary experiments on 21 rabbits and 42 wounds are shown in Table I. It is seen that urea retards the rate of epithelization but stimulates the rate and extent of formation of granulation tissue in proportion to the concentration of urea. 40%, 10%, and 2% levels of urea retard the average rate of epithelization 108%, 16%, and 20%, respectively; whereas the 40% and 10%

TABLE I.

Effect of Urea on Healing of skin wounds in rabbits.

A. *Size of wounds* (Avg cm diam. between epith. boundaries at center of wound).

				Diameter of wounds (cm)			
				Avg original diam. (calc'd from 1st tracings)		Final diam. (meas'd from photographs of sections†)	
No.	% urea	No. of wounds	Days from 1st tracing* to autopsy	Controls without urea	Exptls. with urea	Controls without urea	Exptls. with urea
1‡	40	7	10	1.65	1.55	0.87	1.69
2	10	10	10.3	1.40	1.45	0.82	1.05
3	2	5	11	1.68	1.56	1.03	1.05

B. *Rate of epithelization* (mm per day mean rate of epithelial closure from 1st tracing, 48 hrs after op'n to autopsy. (Calc'd from diameters).

				Rate of epithelization (mm per day)				
No.	% urea	No. of wounds	Days from 1st tracing to autopsy	Controls without urea	Exptls. with urea	% diff.	Student's "t" value[6,7]	Probability[6,7]
1	40	7	10	.78	—.14§	—108	5.6	.0008
2	10	10	10.3	.51	.43	— 16	3.9	.0046
3	2	5	11	.61	.49	— 20	3.5	.0250

C. *Rate of granulation* (mm² per day mean rate of formation of granulation tissue, calc'd from planimetric meas. of photographs of cross sections).

				Rate of granulation (mm² per day)				
No.	% urea	No. of wounds	Days from operation to autopsy	Controls without urea	Exptls. with urea	% diff.	Student's "t" value	Probability
1	40	10	13	2.67	3.64	36	3.0	.0134
2	10	9	12.3	1.98	2.36	19	4.4	.0018
3	2	5	13.5	2.90	2.82	— 3	1.1	.3214

*48 hrs after operation.

†The final diameter cannot be accurately measured from cellophane tracings after more than 5 or 6 days from the time of operation, but must be more accurately determined from stained cross sections.

‡The jelly base in Group 1 contained 0.5% sulfanilamide, whereas those in Groups 2 and 3 contained 5% sulfathiazole. (See text for discussion.)

§A negative rate implies that the strong urea caused the wound to increase in size. (See Table I, part A.)

[6] "Student," *Metron*, 1925, **5**, 18 and 26.

[7] Fisher, R. A., *ibid.*, 1925, **5**, 3.

levels promote the rate and extent of granulation 36% and 19%, respectively. 2% urea had an insignificant effect on the rate of granulation tissue formation. 5% sulfathiazole was substituted for the 0.5% sulfanilamide first used in the experiments with 40% urea, since it was found to inhibit more efficiently bacterial growth. This substitution had no effect on the rate of healing, permitting comparisons to be drawn between the 3 experimental groups. Deletion of the cases which were shown to be significantly infected had no essential effect on the results.

Discussion. It is possible that these results may be explained by an inflammatory action of the urea. This might then result in marked production of granulation tissue from the wound-base, subsequent contraction of this tissue, resultant impaired vascular supply to the surface, and eventual retardation of epithelization as well as formation of overgrowths by fibroplastic stimulation.[8] This concept is supported by Menkin's[9] observations that strong urea is an inflammatory irritant in rabbit tissue. Maceration in our wounds was not apparent.

Because of the delicacy of rabbit tissue, caution must be exercised, as has been pointed out by MacKay,[10] in the extension of these results to other species. Our results suggest that if strong urea is used in the chemical debridement of infected wounds, its use after clean granulation is initiated should be tempered with caution.

Summary. Urea retards the rate of epithelization of open skin wounds of rabbits, but stimulates the rate and extent of formation of granulation tissue; 40%, 10%, and 2% levels of urea in a glyceride jelly base containing sulfanilamide or sulfathiazole retard the average rate of epithelization 108%, 16%, and 20% respectively; 40% and 10% levels of urea promote the rate and extent of granulation 36% and 19% respectively, and 2% urea has little or no effect.

8 Arey, L. B., *Physiol. Rev.*, 1936, **16**, 327.

9 Menkin, V., *J. Exp. Med.*, 1932, **56**, 157.

10 MacKay, E. M., *Arch. Dermatol. Syphilol.*, 1940, **41**, 736.

13578

A Modification of the Method of Hollander and Jemerin for Making Pavlov Pouches.

R. A. Gregory,* George A. Hallenbeck and Charles F. Code.
From the Division of Experimental Medicine, Mayo Foundation, Rochester, Minn.

On the basis of their study of the distribution of the vagus nerves to the stomach of the dog, Hollander and Jemerin[1, 2] developed a technic of making the Pavlov type of gastric pouch. They found fewer vagal nerve fibers were divided in their procedure than in the original Pavlov operation.[3] Jemerin and Hollander emphasized the finding that in the dog, as in man, both vagus nerves course along the lesser curvature of the stomach. From the left vagus nerve, which lies anteriorly, branches traverse the anterior wall of the stomach toward the greater curvature while, from the right vagus nerve, branches supply the posterior wall as they pass toward the greater curvature.

As a result of these studies, new technics for making Pavlov pouches have been developed in the last few years. Cope, MacMahon, Hagströmer and Thompson,[4] having confirmed the findings of Hollander and Jemerin with respect to the distribution of the vagus nerves to the stomach, developed a two-stage operation for making a pouch with an open but non-leaking stoma. Neuwelt, Olson and Necheles[5] have presented a simplification of the Hollander-Jemerin procedure in which, however, four-fifths of the vagal nerve supply to the anterior wall of the pouch is sacrificed. We wish to present another modification of the Hollander-Jemerin technic as applied to the dog, which we have found renders the operation less difficult and which preserves most of the vagal nerve supply to the anterior wall of the pouch and all of the supply to the posterior wall of the pouch.

---

*Fellow Rockefeller Foundation. Present address, University College, London, Near Leatherhead, Surrey, England.

1 Jemerin, E. E., and Hollander, Franklin, Proc. Soc. Exp. Biol. and Med., 1938, **38**, 139.

2 Hollander, Franklin, and Jemerin, E. E., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 87.

3 Pavlov, I. P., *The Work of the Digestive Glands*, J. B. Lippincott Company, 1910.

4 Cope, Oliver, MacMahon, C. E., Hagströmer, Anders, and Thompson, R. H., *Arch. Surg.*, 1940, **40**, 717.

5 Neuwelt, F., Olson, W. H., and Necheles, H., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 74.

With the dog under ether anesthesia and with aseptic technic, the abdominal cavity is entered either through an incision in the median line or through an oblique incision passing about 2 cm caudal to the left costal margin and dividing the left *rectus abdominis* muscle and the abdominal musculature lateral to it. The spleen is delivered to the outside and wrapped in a warm, moist towel. The stomach is drawn into the wound and a rubber booted clamp is placed across it, close to the lesser curvature and parallel to the long axis of the dog (Fig. 1 b). After the rest of the peritoneal cavity has been protected by gauze packs, a curved incision is made in the stomach parallel to the greater curvature and about 1 or 2 mm from the point of disappearance of the gastro-epiploic vessels into the muscular wall of the organ. As a rule this incision does not extend beyond the corpus of the stomach. It should stop 1 cm or more to the oral side of the antrum and usually need not be carried into the fundus. To control bleeding the larger gastro-epiploic vessels, as they lie between the muscle and the mucosa, are clamped in small hemostats. This incision, placed as it is, near the greater curvature, leaves the vagal nerve supply to most of the anterior wall of the pouch intact and that to the posterior wall untouched.

The next step in the operation is to prepare the septum of mucous membrane which is to separate the main stomach from the pouch (Fig. 1 a). This is done by means of an incision in the mucosa of the stomach, running obliquely downward from the fundus above toward the junction of the corpus and antrum below. The incision is carried through the mucous membrane of the anterior and of the posterior wall of the stomach, with corresponding points on the anterior and posterior segments being equidistant from the greater curvature and a sufficient distance from the lesser curvature to allow room for the passage of food (Fig. 1 a). The incision in the mucosa of the anterior wall is joined superiorly and inferiorly with that of the posterior wall so that at this stage of the operation the incision presents an oval appearance (Fig. 1 c). A cuff of mucous membrane, sufficient to allow easy inversion, is dissected free from the underlying muscle at the edges of the incision. The free edge of the cuff lying nearest the lesser curvature on the anterior wall is joined by the Connell type of suture to the corresponding mucosal edge of the posterior wall, effecting an inversion of mucosa into the main stomach (Fig. 1 c). Then, the remaining 2 mucosal flaps are sutured in such a way that they are inverted into the pouch, completing the formation of a mucosal septum in the stomach (Fig. 1 d). The original incision in the stomach is closed in 2 layers. As a

routine, the pouch has been drained by a number 18 dePezzer rubber catheter (Fig. 1 e). In a few instances the Neuwelt, Olson and Necheles type of stainless steel cannula has been used. The rubber tube or cannula is threaded through the omentum and brought out through the abdominal incision or through a separate stab wound in the abdominal wall. Results have indicated that an exit for the tube or cannula separate from the incision allows more rapid healing of the wound. Care must be taken properly to invert the mucosa and

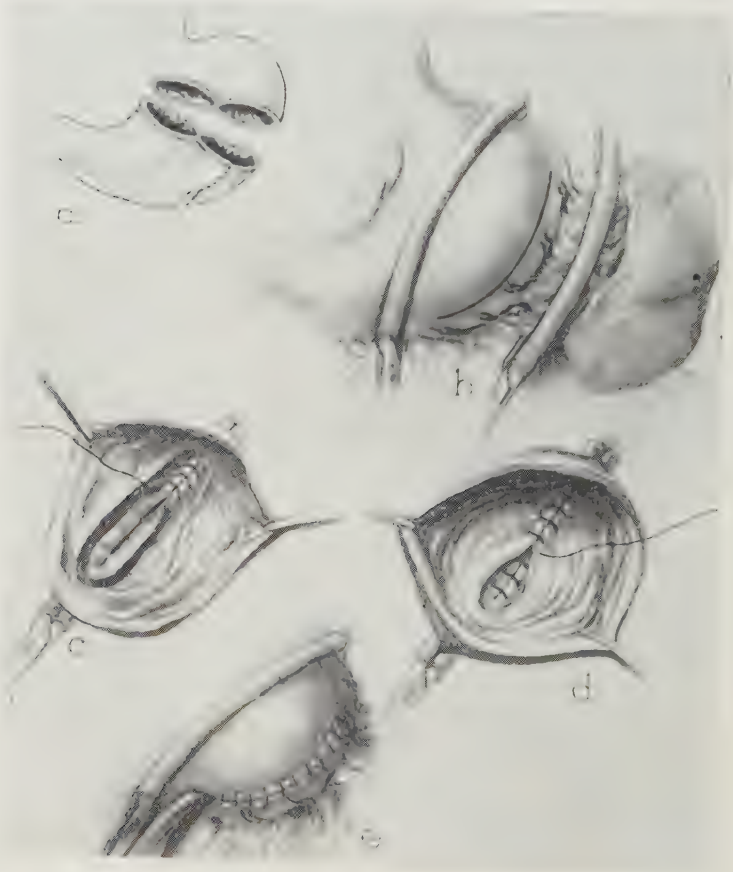


FIG. 1.

Method of preparing Pavlov type of gastric pouch: *a*, cross section of stomach removed to show pouch and main stomach separated by mucosal septum; *b*, incision into stomach; *c*, oval incision in mucosa with posterior edges of mucosa sutured to form one layer of mucosal septum; *d*, oval incision in mucosa with anterior edges partly sutured to complete part of the mucosal septum; *e*, pouch closed and drained with dePezzer catheter. Sutures shown in drawings are diagrammatic. Actually the mucosa is inverted and closed in all instances by a Connell suture and the muscle and serosa of the incision into the stomach are brought together by a simple over and over suture.

muscle around the catheter or cannula. The abdominal incision is closed in two layers; the peritoneum with linen and the fascia with number 1 chromic catgut.

Postoperatively the animals often molest the dePezzer catheters and at times it has been necessary to place a new catheter in the pouch. This complication can be avoided by directing the catheter into a rubber balloon to catch the material which drains forth and protecting the balloon and catheter with a basket strapped to the animal. In this study, however, the basket and balloon were seldom used. Our experience indicates that the presence of the soft rubber catheter in Pavlov or Heidenhain pouches is well tolerated. Fasting values of 0.0 to 0.05 cc of juice per half hour, with no free acid present, are obtained routinely from pouches drained by rubber catheters. Observations in the course of this investigation on pouches drained by the Neuwelt, Olson and Necheles stainless steel cannulas have been too limited to justify conclusions.

In response to a meat meal a Pavlov pouch, prepared by the procedure outlined in this report, quickly yields a highly acid gastric juice (Table I).

Summary. By modification of the Hollander-Jemerin technic of making a Pavlov type pouch, the vagal nerve supply to most of the anterior wall of the pouch, and to all of the posterior wall, is preserved.

TABLE I.

Responses of a Pavlov Pouch in a Dog Weighing 7 kg to 7 Feedings of 100 g of Raw, Lean Horse Meat. Samples Collected at Half-hour Intervals Before and After Feeding.

Half-hour samples	Volume of juice, cc			% free HCl in juice			Avg HCl output, mg per hr
	Max.	Min.	Avg	Max.	Min.	Avg	
Control	0.6	0.0	0.18	.0	.0	.0	0.0
1	14.9	7.0	10.2	.551	.408	.476	49.6
2	21.3	13.6	17.8	.602	.537	.569	102.0
3	21.3	13.7	16.6	.602	.569	.581	96.7
4	15.6	11.7	13.5	.598	.572	.581	78.5
5	13.4	9.2	10.8	.613	.562	.579	62.3
6	10.8	6.5	7.8	.584	.558	.570	44.2
7	6.1	3.5	5.2	.555	.537	.546	28.1
8	5.0	1.8	3.2	.533	.486	.512	17.4
Total 4-hour output			85.1				

13579

Effect of Low Atmospheric Pressure on the Adrenals, Thymus and Testes of Rats.

F. C. Dohan.

From the George S. Cox Medical Research Institute, University of Pennsylvania, Philadelphia.

Recent metabolic[1-4] and histologic[4] studies indicate that the adrenal cortex plays an important rôle in the response of the mammalian organism to lowered oxygen tension in the inspired air. In addition, an increase in the ratio of adrenal weight to body weight in rabbits exposed to ½ atmosphere for 4 hours daily over a period of weeks has been reported by Armstrong and Heim.[5]

The following experiments demonstrate an increase in the *absolute* weight of the adrenal glands in association with a decrease in weight of the thymus and testes in rats exposed to low atmospheric pressure on more than 2 days. This pattern of response is similar to that found by Selye[6, 7] in animals subjected to other forms of stress ("alarm reaction"). We have also observed that diets of widely varying composition had no significant effect upon this change of weight of the glands.

Methods. Male white rats (Sprague-Dawley) were used. The body weight ranged from 270 to 345 g with the exception of one rat weighing 372 g. After a preliminary observation period of 5 to 10 days during which the animals were fed enough of the special diets indicated below to maintain their body weights, they were placed in a low pressure chamber* for small animals and maintained at the various pressures for various periods. The pressure in the chamber was lowered by a pump and kept at approximately the desired level by means of an accessory inlet valve. This was controlled electrically by a closed mercury manometer connected to the chamber. At the

1 Evans, G., *Am. J. Physiol.*, 1934, **110**, 273.

2 Evans, G., *Am. J. Physiol.*, 1936, **114**, 297.

3 Lewis, R. A., Thorn, G. W., Koepf, G. F., and Dorrance, S. D., *J. Clin. Invest.*, 1942, **21**, 33.

4 Giragozsints, G., and Sundstroem, E., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 432.

5 Armstrong, H. G., and Heim, J. W., *J. Aviat. Med.*, 1938, **9**, 92.

6 Selye, H., *Endocrinology*, 1937, **21**, 169.

7 Selye, H., *Canad. M. A. J.*, 1940, **42**, 113.

* The low pressure chamber was kindly loaned to us by Dr. C. N. H. Long and Dr. Gerald T. Evans.

lower pressures used the animals developed cyanosis and dyspnea. The pressure was decreased to a constant level over a period of approximately 5 to 15 minutes. Before the animals were removed from the chamber the pressure was increased to atmospheric level in about 2-5 minutes. No significant effects were noted upon the gland weights of 2 rats kept in the pressure chamber only during these periods of adjustment.

The special diets were a slightly modified form of those described by Reinecke, Ball and Samuels[8] for tube feeding. They contain approximately 300 calories per 100 ml. The protein diet was composed largely of casein and could not be tube fed. The caloric distribution of the diets was calculated to be as follows:

	%	%	%
High carbohydrate diet	P 18	F 10	C 72
" fat diet	P 18	F 81	C 1
" protein diet	P 89	F 10	C 1

Normal adult rats maintained their body weight when offered any one of these diets. When fed by tube, a total of 20 ml per day maintained the body weight when at atmospheric pressure. Special precautions were taken not to induce an alarm reaction by too rapid an increase in the amount of food given by tube.[9] The possible effect of this factor was controlled by (1) tube feeding 2 of the control group with no significant effect upon the gland weights, (2) feeding one experimental group a diet to be eaten *ad libitum* and (3) an experiment with starved animals.

The adrenals, thymus and testes of the rats dying during the period in the low pressure chamber as well as those surviving this period were weighed. The surviving animals were anesthetized with ether and then bled to death. Fisher's small sample technic[10] was used for the statistical analysis of the results.

Results. Details concerning the diet, pressure and survival are given in Table I and the legend of Fig. 1. The gland weights are charted in Fig. 1. The probability that the differences in the means of the experimental groups from those of the control group (A) were due to chance is indicated in Table II, in which we have taken as significant those differences for which the probability of occurrence by chance is not greater than 0.05. It is evident that groups C, D and E show a significant increase in mean adrenal weights and decrease in

8 Reinecke, R. M., Ball, H. A., and Samuels, L. T., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 44.

9 Ingle, D. J., *Endocrinology*, 1941, **29**, 838.

10 Fisher, R. A., *Statistical Methods for Research Workers*, Oliver and Boyd, London, 1938.

TABLE I.
Survival, Diet and Pressure.

		Number of rats dying on day and at pressure indicated*							
Group C (11 rats)	Day	1	2	3	4	Survived			
	Pressure†	355	315	275	255-235				
Diet: Tube fed	CHO		3			2			
2 × 10 ml daily	Fat		1		1	4			
Group D (6 rats)	Day	1	2	3	Survived				
	Pressure	293	293	293					
Diet: Tube fed	CHO	2			1				
1 × 13 ml daily	Fat	2		1					
Group E (7 rats)	Day	1-2	3-7	8-9	10	11	12	13	Survived
	Pressure	380-340	300	275	255	255	235	235	
Diet: ad libitum	CHO							1	2
	Fat							1	1‡
	Protein				2				

*Groups C and D were in the low pressure chamber 22 to 23 hours daily. Group E was in the chamber about 15½ hours daily. The diet was offered only when out of the chamber.
†Pressure in mm of Hg.
‡Sacrificed on 13th day.

mean thymic weights. The differences of the mean weight of the testes from that of the control group is significant in all except

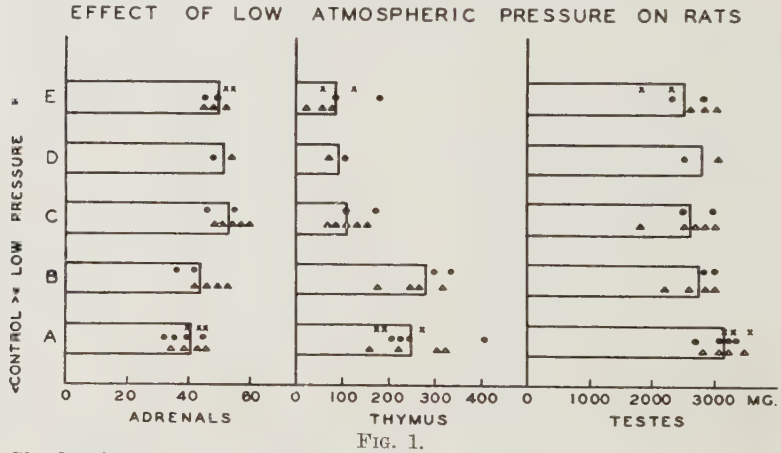


FIG. 1.

Gland weights in rats receiving:
● = high carbohydrate diet
△ = high fat diet
× = high protein diet

Group A—Control group. Atmospheric pressure. Diets eaten *ad libitum* except for 2 rats fed by tube.

Group B—Is composed of rats in Group C and Group D of Table I which died within 2 days after placing in low pressure chamber. Two of the rats in Group C (Table I) which died on the 2nd day were discarded without weighing the glands.

Group C—In this figure only those rats of Group C, Table I, which survived longer than 2 days are charted.

Group D—In this figure only those rats of Group D, Table I, which survived longer than 2 days are charted.

Group E—Same as Group E, Table I.

TABLE II.
Probability That the Difference Between Mean Organ Weights Shown in Fig. 1 Might Have Occurred by Chance.

Group	Glands	Diff. of means of organ wts, mg	Stand. error of diff., mg	Probability of chance occurrence of diff.,* P
B-A	Adrenals	+ 3.7	2.3	>.10†
	Thymus	+ 37	34.3	>.20†
	Testes	—430	124	<.01
C-A	Adrenals	+ 13.1	2.1	<.01
	Thymus	—146	30.2	<.01
	Testes	—570	148	<.01
D-A	Adrenals	+ 15.5	3.4	<.01
	Thymus	—155	53.4	<.02
	Testes	—330	192	>.10†
E-A	Adrenals	+ 10.0	1.8	<.01
	Thymus	—158	33.2	<.01
	Testes	—600	149	<.01

*From Fisher's table of t values.
†Difference not statistically significant.

Group D, consisting of only 2 rats. Those animals dying within the first 48 hours (Group B) do not show a significant deviation of the adrenal or thymus gland weights from the control group. However, the difference in the weight of the testes is significant.

The distribution of the data (Fig. 1) does not indicate a significant *effect of diet* upon the gland weights either at atmospheric pressure or below; nor could any differential effect of diet upon survival be obtained from analysis of our small number of data (Table I). Thus, combining the results in Groups C and D, 3 of 8 rats fed high

TABLE III.
Effect of Atmospheric Pressure of Approximately 300 mm Hg. for 3 to 5 Days upon Gland Weights of Starved Rats.

Glands		No. of rats	Mean gland wt, mg	Stand. error of diff. of means, mg	Probability of chance occurrence of diff.*
Adrenals	Control	6	36.9	4.1	<0.02
	Experimental	5	48.6		
Thymus	Control	6	200	15.6	<0.01
	Experimental	5	81		
Testes	Control	6	2934	87	<0.05
	Experimental	5	2719		

*From Fisher's table of t values.

carbohydrate and 4 of 9 rats fed high fat diets survived until the end of the experiments. *In starved animals* a statistically significant qualitatively similar effect upon gland weights has also been obtained (Table III).

Attempts were made to determine the effect of adrenal cortical extracts† upon the change in the adrenal weights. The results were inconclusive using subcutaneous injections as high as 5 cc twice daily.

Discussion. The above data indicate a definite effect of prolonged exposure to low atmospheric pressure upon the absolute weights of the adrenal, thymus and testes. The pattern of change and the general condition of the animals was similar to that which may be produced by cold, injections of formalin or morphine, severe muscular work and other forms of stress (alarm reaction of Selye[6, 7]). The effect of diet upon prevention or amelioration of the "alarm reaction" due to other agents has not been reported. However, in connection with the lack of effect of diet in our experiment it is well to point out the long time intervals between feedings.

Summary. Rats exposed to low atmospheric pressure for more than 2 days exhibited a significant increase in adrenal weight and a significant decrease in the weight of the thymus and testes. This pattern is similar to that found following other forms of stress. No significant effect of diet upon this change was demonstrated.

13580 P

A Method for Inducing Limb Regeneration in Adult Anura.

S. MERYL ROSE. (Introduced by Otto Glaser.)

From the Department of Biology of Amherst College.

It has been demonstrated by Barfurth[1] and confirmed by subsequent investigators that frogs apparently lose their ability to regenerate limbs during the tadpole stage. On the other hand, a few adult frogs[2] and toads[3] have been observed with very definite indications of limb regeneration. These few cases of regeneration in adults are rare. Usually the stumps heal without regenerating.

† We are indebted to Dr. George F. Cartland of the Upjohn Company for the adrenal cortical extract.

[1] Barfurth, D., *Arch. f. Entw.-Mech.*, 1894, **1**, 117.

[2] Gadow, H., *Amphibia and Reptiles*, The Cambridge Natural History Series, 1901.

[3] Woodland, W. N. F., *Q. J. M. S.*, 1921, **65**.

A method of inducing limb regeneration in tadpoles too old to regenerate, after simple amputation, has been described by Polejaiev.[4] His method involves injury to the stump by cutting. Since *Tubularia* does not regenerate unless a cut surface is maintained where exchanges occur between tissue and environment[5] and since urodele limbs fail to regenerate when the amputation surface is covered by epidermis,[6] the following experiment was performed. The normal irritation following amputation was prolonged in Anura by baths in a saturated sodium chloride solution.

Parts of limbs were removed from *Rana clamitans* varying in stage from a few weeks past metamorphosis to full-grown. Twenty-two amputations were made between elbow and wrist or through the hand. All frogs were kept in water. Experimental animals were bathed in saturated sodium chloride solution twice daily for several minutes. The treatments continued for from 1 to 8 weeks with different animals. Fifteen control animals were not treated with salt.

The effects of the treatment were quite pronounced. The stump reddened, the color persisting for several hours, in some cases until the next treatment. The wounds of some of the animals bled during

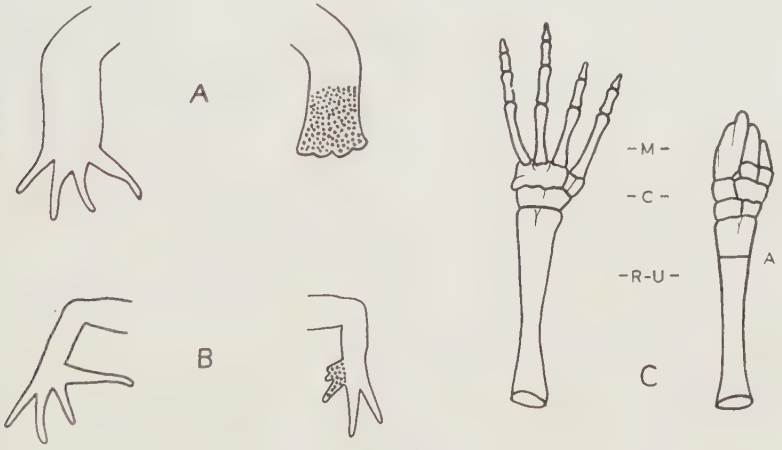


FIG. 1.

Comparison of Salt-induced Regenerants and Normal Limbs.

A—Regenerant stippled. Amputation 7 months earlier when frog was a year past metamorphosis. Treated for 8 weeks.

B—Amputation 4 months earlier when frog was 1 month past metamorphosis. Treated for 3 weeks.

C—Skeletons of regenerant and normal arm compared. A, level of amputation. Regenerated skeletal elements: R-U, part of radio-ulna; C, carpals; M, metacarpals.

[4] Polejaiev, L. W., *Arch. d'Anat. Micr.*, 1936, **32**, 437.

[5] Rose, S. M., and Rose, F. C., *Physiol. Zool.*, 1941, **14**, 328.

[6] Schaxel, J., *Auffassungen und Erscheinungen der Regeneration*, Berlin, 1921.

the salt treatments of the first 2 days; all remained open longer than those of the controls.

Of the 22 experimental animals, only one failed to regenerate. None of the limbs of the 15 controls developed past the stage of a healed stump. The blastema on the limbs of the frogs whose arms were removed below the elbow began as a single mound of soft tissue. The single mound grew and differentiated into a wedge of tissue with the thin edge at the distal border. As growth proceeded a cleft converted the wedge into a double structure distally. Some of the regenerants developed further with new clefts appearing in each of the distal protuberances. By this time it was apparent that the resulting 4 protuberances were finger buds. Fig. 1 A shows such a regenerant. Alongside the regenerant is shown the corresponding limb of the other side. This animal was 5.2 cm long at the time of limb amputation in July and had metamorphosed the previous year or earlier. Fig. 1 B shows finger regeneration following amputation of two fingers from a first year frog. The regenerated first finger appears to be double and the second finger normal. Fig. 1 C shows the regenerated skeletal elements in a frog whose arm had been amputated through the radio-ulna. A movable wrist and imperfect hand regenerated. Skeletal elements along with other internal structures of the regenerants will be described elsewhere.

Adult frog limbs, therefore, regenerate when the wound is kept irritated with a strong salt solution.

13581

Nitrogen Content and Total Osmotic Activity of Gastric Juice.*

NATHAN LIFSON, RICHARD L. VARCO AND M. B. VISSCHER.

From the Department of Physiology, University of Minnesota.

In a previous communication[1] we reported the degree of hypo- or hypertonicity of gastric juice samples collected from chronic greater curvature (Heidenhain) and pyloric pouches in dogs. Histamine stimulated juices were 2-6 m.E./K hypertonic; pilocarpine stimulated juices were either iso- or hypotonic; and fasting juices were hyper-,

* We are indebted to Mr. Charles Carr for carrying out many of the chemical determinations for this study.

1 Lifson, Nathan, Varco, Richard L., and Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 422.

hypo-, or isotonic. Since pilocarpine results in more mucin production than does histamine, it seemed of interest to ascertain whether an inverse correlation existed between the nitrogen content and osmotic activity of these same secretion samples. The material studied in our previous work[1] was, therefore, used for the study reported here.

Methods. The osmotic activity data are the same reported in the previous paper, in which the experimental procedure, and analytical methods involved in the study of 31 samples from 7 dogs, have been described.[1] Free acid determinations were made directly upon collection; but the total nitrogen, chloride and total fixed base were measured on the same material after storage at freezing temperature for 60-100 days. Total nitrogen was estimated by the micro-Kjeldahl method without preliminary removal of constituents by centrifuging, filtering, or similar means. Free acidity was determined with dimethyl-amino-azo-benzene (Topfer's reagent) as the indicator. Total fixed base was measured on ashed samples by the electrodialysis method of Keys and Adair[2] as modified by Consalazio and Talbott.[3] Chlorides were done by a micro-modification of the Volhard titration.

Results and Discussion. A. *Osmotic Activity and Nitrogen Content.* Tables I and II contain data for 30 samples, the following features of which will be noted:

(1) For greater curvature pouch samples, the histamine-stimulated juices had a total nitrogen content of 50 mg per 100 cc or less, whereas the pilocarpine-stimulated juices all had a total nitrogen content of 65 mg per 100 cc or more. Fasting samples possessed values in both ranges.

(2) Pyloric pouch juices had a total nitrogen content at least 100 mg per 100 cc higher than any of the greater curvature pouch juices.

(3) Samples with approximately the same total nitrogen content and collected under comparable circumstances may exhibit relatively large differences in osmotic activity. Compare, for example, Exp. VIIa with Exp. XVIa, or VIb with XIIb. (Table I.)

(4) Samples with approximately the same osmotic activity and collected under comparable circumstances may exhibit relatively large differences in total nitrogen content. Compare, for example, Exp. IIIa with Exp. XIIa. (Table I.)

The anticipated inverse correlation between total nitrogen content

2 Adair, G. S., and Keys, A. B., *J. Physiol.*, 1934, **81**, 162.

3 Consalazio, W. V., and Talbott, J. H., *J. Biol. Chem.*, 1940, **132**, 753.

TABLE I.

Exp. No.	Condition of collection	Site of pouch	Osmotic activity of Plasma, m.E./K. H_2O	Osmotic activity of Gastric juice, m.E./K. H_2O	OA (Osmotic activity of gastric juice minus plasma), m.E./K. H_2O	Total nitrogen of gastric juice, mg per 100 cc
IIa	Fasting	Greater Curvature	157	155	— 2	50
IIIa	”	” ”	158	156	— 2	8
Va	”	” ”	155	151	— 4	63
Xa	”	” ”	158	156	— 2	95
VIIa	”	” ”	163	151	—12	67
VIIIa	”	” ”	161	161	0	70
IXa	”	” ”	161	167	+ 6	180
XIIa	”	” ”	161	158	— 3	95
XIVa	”	” ”	157	157	0	33
XVIa	”	” ”	161	161	0	60
XIa	”	Pyloric	157	161	+ 4	340
XIIIa	”	”	161	165	+ 4	500
XVa	”	”	157	169	+12	350
XVIIa	”	”	161	162	+ 1	280
Ib	Histamine	Greater Curvature	159	161	+ 2	28
IIIb	”	” ”	161	167	+ 6	12
IVb	”	” ”	156	162	+ 6	50
Vb	”	” ”	152	155	+ 3	21
Xb	”	” ”	160	163	+ 3	30
VIb	Pilocarpine	” ”	160	150	—10	90
VIIb	”	” ”	162	154	— 8	160
VIIIb	”	” ”	161	145	—16	90
IXb	”	” ”	163	147	—16	160
XIIb	”	” ”	162	162	0	100
XIVb	”	” ”	163	162	— 1	65
XVIb	”	” ”	165	165	0	130
XIb	Histamine	Pyloric	160	164	+ 4	500
XIIIb	Pilocarpine	”	162	153	— 9	400
XVb	”	”	163	161	— 2	400
XVIIb	”	”	165	164	— 1	400

TABLE II.

Conditions of collection and type of pouch	No. of cases	OA Mean m.E./K. H_2O	OA Range m.E./K. H_2O	Total nitrogen of gastric juice Mean mg per 100 cc	Total nitrogen of gastric juice Range mg per 100 cc
Fasting, Greater Curvature	10	—1.9	—12 to + 6	72	8-180
Histamine, ” ”	5	+4	+ 2 to + 6	28	12-50
Pilocarpine, ” ”	7	—7.3	—16 to 0	104	65-160
Fasting, Pyloric	4	+5.2	+ 1 to +12	368	280-500
Histamine ”	1	+4		500	
Pilocarpine, ”	3	—4	— 1 to — 9	400	

and osmotic activity appears most clearly in the comparison between histamine- and pilocarpine-stimulated juices obtained from greater curvature pouches. The former juices all were higher in osmotic activity and lower in total nitrogen content than any of the latter. In this connection it may be mentioned that Gilman and Cowgill[4] showed that in histamine-stimulated secretions from Pavlov pouches, hypotonicity up to 17 m.E./K. was associated with high mucus content. Our results indicate. however, that the relationship between the two variables measured is not a close one, especially if all the samples are considered as a single group without regard to conditions of collection or type of pouch. The nitrogenous constituents of gastric juice, by virtue of their buffer capacity, could participate in the production of hypotonicity in a manner previously described.[5, 1] The absence of closer correlation between total nitrogen content and osmotic activity may be explained in part by (a) the fact that total nitrogen is probably not an accurate index of this nitrogenous buffer capacity,[6] and in part by (b) the operation of quantitatively more important factors, in particular, buffering by non-nitrogenous constituents.

B. *Comparison of Calculated and Observed Osmotic Activities.* Table III contains the results of determinations of free acidity, total fixed base, and chlorides, all expressed in m.E. per liter of gastric juice, for nine of the samples, arranged in the order of increasing total nitrogen content. From these additional data, the total osmotic activity of the samples in question has been calculated on the assumptions that (1) free acidity is equivalent to hydrogen ion concentration, (2) all the fixed base is univalent, (3) the difference in units employed is negligible, *i. e.*, non-solvent volume has been ignored, and (4) no other osmotically active constituents are present. It will be noted in Table III that for the six samples in which free acid occurred (all from greater curvature pouches) the agreement between calculated and observed osmotic activity values is reasonably close, *i. e.*, within 6 m.E./K. However, for the 3 samples with no free acid (all from pyloric pouches) the calculated values are 12, 24, and 24 m.E./K., respectively, less than the observed values. Such discrepancies as the last two suggest that one or more of the assumptions involved in the calculation may be erroneous. But the first of these assumptions appears to be exonerated by the absence of free

4 Gilman, Alfred, and Cowgill, George R., *Am. J. Physiol.*, 1933, **103**, 143.

5 Hollander, Franklin, *Am. J. Dig. Dis. and Nut.*, 1936, **3**, 651.

6 Bonis, A., *Z. f. Klin. Med.*, 1930, **113**, 611; Mitchell, T. Carlyle, *J. Physiol.*, 1931, **73**, 427.

TABLE III.

Comparison of Calculated and Observed Osmotic Activities of Gastric Pouch Juices.

Exp. No.	Condition of collection	Site of pouch	Free acidity, m.E./L.	Total fixed base, m.E./L.	Chlorides, m.E./L.	Calculated osmotic activity, m.E./L.	Observed osmotic activity, m.E./K. H_2O	Calculated minus observed	Total nitrogen content, mg/100 cc
Xb	Histamine	Greater Curvature	141	15	170	163	163	0	30
XIVa	Fasting	"	141	25	156	161	157	+ 4	33
XVIa	"	"	144	24	165	166	161	+ 5	60
VIb	Pilocarpine	"	122	21	159	151	150	+ 1	90
XVIb	"	"	152	19	167	169	165	+ 4	130
IXa	Fasting	"	2	160	168	165	167	— 2	180
XVa	"	Pyloric	0	160	154	157	169	—12	350
XVb	Pilocarpine	"	0	130	144	137	161	—24	400
XVIIb	"	"	0	124	153	138	164	—24	400

acid, and the second by the fact that any polyvalent base would yield fictitiously high rather than fictitiously low calculated osmotic activities. Correction for non-solvent volume would not account for more than 6-7 m.E./K. However, it may be that the fourth assumption does not hold for anacid juices, particularly of pyloric origin. The high total nitrogen content of such secretion samples suggests that nitrogenous constituents of relatively low molecular weight may account for an appreciable part of the osmotic deficit.

Summary. 1. In general, pilocarpine-stimulated greater curvature gastric pouch juice has a lower osmotic activity, and high total nitrogen content than does histamine-stimulated juice. 2. Fasting juices show highly variable relations between total nitrogen content and osmotic activity. 3. Pyloric pouch fasting juice has in our experience very much higher total nitrogen content than does greater curvature pouch juice. The osmotic activity of the former is nevertheless hypertonic with respect to blood. 4. There appears to be a partial correlation between total nitrogen content and osmotic activity of gastric juice. 5. Calculated and observed osmotic activities are in reasonable agreement for six greater curvature pouch samples in which free acid was present. 6. Calculated values were 12 to 24 m.E./K. lower than the observed osmotic activity values for three pyloric pouch juices without free acid. It is suggested that part of the deficit results from neglecting in the calculation the contribution of the nitrogenous constituents.

13582

An Analysis of Tobacco Mosaic Virus for Biotin, Riboflavin and Pantothenic Acid.

HERBERT SPRINCE AND EMANUEL B. SCHOENBACH. (Introduced by J. F. Enders.)

From the Department of Bacteriology and Immunology, Harvard Medical School and School of Public Health, and the Cancer Commission of Harvard University, Boston, Mass.

Investigation into the structure of filterable viruses has revealed the presence of some amino acids,[1, 2] certain enzymes such as catalase

[1] Ross, A. F., *J. Biol. Chem.*, 1941, **138**, 741.

[2] Knight, C. A., and Stanley, W. H., *J. Biol. Chem.*, 1941, **141**, 39.

and phosphatase,[3, 4] as well as other important biologically active compounds, notably biotin and riboflavin.[5, 6] The presence of these latter growth-promoting substances has been demonstrated only in a highly organized virus, namely, the elementary bodies of vaccinia.[5, 6] It, therefore, became of interest to determine whether less complex viruses, such as tobacco-mosaic virus which could be obtained in a highly purified form might contain such substances. Investigation of such a virus for the presence of highly active chemical groupings might well furnish valuable information concerning the metabolic relationship between the virus molecule and host-tissues. Accordingly, highly purified tobacco-mosaic virus was examined for the presence of biotin, riboflavin, and pantothenic acid. Microbiological methods of assay for these vitamins are extremely sensitive and determinations can be made with a reliable degree of precision.

Methods. A sample of tobacco-mosaic virus suspended in 0.1 M total phosphate buffer pH 7.0 was obtained through the kindness of Professor E. J. Cohn of the Department of Physical Chemistry, who received it from Dr. Lauffer of the Rockefeller Institute for Animal Research at Princeton. A five-fold dilution of this material was then assayed for riboflavin by the method of Snell and Strong[7] and for pantothenic acid by the method of Pennington, Snell and Williams.[8] Aliquots of a five-fold dilution of unhydrolyzed virus and virus hydrolyzed with 5% HCl for 1 hour at 15 lb pressure, neutralized and brought to pH 4.5 were also tested for biotin by the procedure of Snell, Eakin and Williams.[9]

Results. No evidence of the presence of riboflavin or pantothenic acid in unhydrolyzed solutions of tobacco-mosaic virus was obtained. Biotin, however, was present in appreciable amounts and further work was then undertaken to investigate its relationship to the virus molecule.

The virus solution was filtered through a graded collodion-filter (540 mu) to remove certain bacteria found to be present, and then

[3] Macfarlane, M. G., and Salaman, M. H., *Brit. J. Exp. Path.*, 1938, **19**, 184.

[4] Macfarlane, M. G., and Dolby, P. E., *Brit. J. Exp. Path.*, 1940, **21**, 219.

[5] Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 669.

[6] Hoagland, C. L., Ward, S. M., Smadel, J., and Rivers, T. M., *J. Exp. Med.*, 1941, **74**, 133.

[7] Snell, E. E., and Strong, F. M., *Ind. Eng. Chem. Anal. Ed.*, 1941, **11**, 346.

[8] Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **135**, 213.

[9] Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 175.

thrown down in the ultracentrifuge (800 rps for 15 minutes). The supernatant fluid was removed, the virus washed once with distilled water, and again deposited by centrifugation. During the ultracentrifugation, the presence of a single sedimentation-peak gave definite indication of the purity of the virus preparation. A micro-Kjeldahl determination of the filtered virus material, before ultracentrifugation, gave a value of 1.37 mg of nitrogen per ml.

Several fractions were obtained after ultracentrifugation, namely, the original supernatant fluid, the supernatant fluid after the first washing, and the resuspended, centrifuged, washed virus. Assay of each component for biotin revealed, as may be seen in Table I, that the major portion of this substance remained in the *original* supernatant fluid and accordingly was not intimately associated with the virus.

A small amount of biotin was present in the washed, resuspended virus. This, we believe, may have been due to slight residual retention of the original supernatant fluid by the virus, probably by absorption. No increase in biotin was noted upon pressure-hydrolysis of the virus. An increase would presumably have occurred if the biotin were an integral part of the virus molecule.

Although it has been demonstrated that biotin and riboflavin are both present in high concentrations in the purified elementary bodies of vaccinia, we have been unable to obtain evidence for their presence in significant amounts in the purified tobacco-mosaic virus. We believe that the large amount of biotin found in the original supernatant fluid of the preparation we examined probably was

TABLE I.
Biotin Assay of Centrifuged Tobacco Mosaic Virus.

	Micrograms $\times 10^{-6}$ biotin per 1.37 mg of nitrogen
(1) Virus sol'n, filtered, centrifuged, but *not* hydrolyzed	162
(2) Virus sol'n, filtered, centrifuged, and hydrolyzed	143
(3) Original supernatant fluid (obtained by centrifuging the filtered virus)	835
(4) Supernatant from the first H_2O washing	93
(5) Sum total of (2), (3) and (4)	1071
(6) Virus sol'n, filtered and hydrolyzed, but *not* centrifuged	1044

Notes: (a) The large amount of biotin found in (3) indicates that the major portion was present in the original fluid in which the virus was dissolved and was not intimately associated with the virus.

(b) The close check between (1) and (2) indicates that no increase in biotin resulted on hydrolysis of the virus. This is further confirmation that biotin is not intimately associated with the tobacco-mosaic virus molecule.

(c) The sum total of (2), (3) and (4) checks very well with (6) indicating that the experimental error of the method was less than 3%.

derived either from the original tobacco-plant material or from certain bacteria which were isolated from the solution of virus before filtration through the collodion filter. Accordingly, we suggest that great caution must be observed in interpreting the significance of analyses of materials derived from living tissues when subjected to delicate procedures of assay.

Conclusions. The presence of riboflavin, pantothenic acid, and biotin could not be demonstrated in significant amounts in moderately concentrated solutions of purified tobacco-mosaic virus.

We are deeply indebted to Dr. Oncley and Mr. Gordon, of the Department of Physical Chemistry, for performing the ultracentrifugation, to Dr. Robinson, of the Massachusetts State Antitoxin and Vaccine Laboratory, for the micro-Kjeldahl determination, and to Dr. Enders, of the Department of Bacteriology, for helpful suggestions in this investigation.

This work was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

13583

Sulfanilylguanidine in Control of Salmonella Infection and Carrier State in Mice.

Parker R. Beamer. (Introduced by Robert A. Moore.)

From the Department of Pathology, Washington University School of Medicine, St. Louis, Mo.

The presence of an enzootic Salmonella infection in our laboratory colonies of mice has prompted an investigation into the etiology, control, and nature of this disease. Data gathered from animal autopsies and bacteriological studies indicate that in 2 strains the infection follows different courses. The 2 strains of mice, Akh and Rfi, were developed by Furth of Cornell University Medical School for his studies of leukemia. Seventy to 80% of the Akh animals die of lymphoid leukemia if they live to be over 8 months of age. The Rfi mice carry myeloid leukemia and 1% die of the disease. Bacteriological studies of stool cultures revealed a significantly higher incidence of infection in Rfi mice than in Akh animals. Autopsies performed on 26 Akh mice in a 2-months' period revealed gross evidence of Salmonella infection in only 2, whereas gross evidence of this disease was seen in 17 animals in a series of 35 Rfi mice autopsied during the same period. The typical lesions seen at autopsy were numerous, irregularly distributed, white areas of focal necrosis, variable in

shape, 0.5 to 5 mm in diameter, occurring most frequently in the liver and spleen. Small hemorrhagic areas and minute ulcerations were occasionally present in the intestinal tract. In every instance where attempted, an organism identified as *Salmonella enteritidis* was isolated from the lesions in both strains of mice. On the basis of preliminary studies, to be completed and reported later, the organisms isolated from both strains of mice appeared to be identical.

Cultures from individual animals were made as follows: Fecal pellets were collected directly from the animals on sterile swabs and emulsified in 2 cc of sterile tryptose broth before streaking on brilliant green lactose agar plates[1] (extract agar (pH 7.0), 1% lactose, 0.1% dextrose, 1% Andrades indicator, brilliant green 1-250,000). Plates were incubated at 37°C and observed at intervals for 48 hours. Typical organisms were identified by fermentation reactions in several carbohydrate media and confirmatory microscopic agglutination tests were performed. The mice were kept in enamel pans with wire covers. The food was suspended in a wire container and water was supplied in an 8 oz bottle. The water bottles were sterilized once a week. The bedding was wood-shavings and the pans were cleaned every 4 to 7 days.

The incidence of infection in the colony as a whole was based on one plate culture. This is not as efficient as a series of cultures, since results of this investigation and others reported in the literature show that, although many mice are continuous excreters of the organisms, others excrete the organisms intermittently. Thirty-five pairs of Akh breeding mice showed an incidence of 31% (males 26%, females 37%). There were 18 pans in which both mice were negative, 12 in which only 1 was positive, and 5 where both mice were positive. Further study with a series of stool cultures for each animal indicated the above figures should be higher, since some of the mice were intermittent excreters. Eight pairs of Rfi breeding mice were available for examination; 13 (81%) of these were positive. The incidence in males and females was not notably different. Among the stock mice, 3 to 10 males or females per cage, the incidence in Akh mice was 14% compared to 50% in Rfi mice.

On the basis of the reports by Marshall and his associates[2, 3] of beneficial results from the use of sulfanilylguanidine in the treatment

[1] Buchbinder, L., Hall, L., Wilens, S. L., and Slanetz, C. A., *Am. J. Hyg.*, 1935, **22**, 199.

[2] Marshall, E. K., Jr., Bratton, A. C., White, K. H., and Litchfield, J. T., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 163.

[3] Marshall, E. K., Jr., Bratton, A. C., Edwards, L. B., and Walker, E., *Bull. Johns Hopkins Hosp.*, 1941, **68**, 94.

of children with bacillary dysentery, it seemed desirable to investigate the effect of this drug in mice infected with *Salmonella enteritidis*. Several pans of Akh breeding mice were cultured serially at 3- to 6-day intervals and 16 pairs showing at least 2 positive plates out of 4 were selected for study. The drug, which is relatively insoluble, was suspended in water so that 0.1 cc contained the desired dose. An initial dose of 12-15 mg was given to one mouse in each cage followed by daily doses of 6-8 mg. Stool specimens collected during the period of chemotherapy and thereafter were cultured on brilliant green lactose agar to which p-amino benzoic acid was added in a final concentration of 10 mg %. During the first 5 days of treatment duplicate plate cultures from 12 of 15 treated mice were negative for Salmonella organisms. Seven to 10 days after starting chemotherapy 2 of the above 12 contained a few Salmonella colonies. The dose of sulfanilylguanidine was then increased to 13 mg and finally to 20 mg daily. These larger doses were given in powder form directly into the pharynx, followed by a few drops of water. After a period of 17 days, during which the stools were cultured in duplicate 4 times on brilliant green lactose agar with p-amino benzoic acid added, 11 of the 15 treated mice showed consistently negative cultures while the incidence of positive stools was unchanged in the untreated mates. Three of the treated mice had consistently positive stools and an additional 1 had only 2 to 6 Salmonella colonies per plate. All the treated mice were isolated into individual cages and the drug was discontinued after 2 days. During the next 70 days frequent stool cultures were made and in only 2 of the negative animals did the stool cultures become positive for Salmonella organisms.

Since the drug appeared to have some beneficial effects, we began treatment of Rfi stock mice by dissolving 150 mg of sulfanilylguanidine in 100 cc of drinking water. On the basis of measured water consumption, it was calculated that each mouse received 6 or more mg of drug daily. A series of 3 stool cultures were made in duplicate at the beginning of the experiment and repeated after 39 days of treatment. If one arbitrarily classifies the mice as +, ++, and +++ on the basis of the number of positive stool cultures in the series of 3, there were: (1) 17 +++ animals treated, 2 of which became negative, 7+, 5 ++, and 3 remained +++; (2) 18 ++ animals treated, 7 becoming negative, 7 +, 3 remaining ++, and 1 changing to +++; (3) 6 + animals treated, 4 of which turned negative and 2 became +++. Thus, it is seen that in most instances the drug appeared to have a beneficial effect, but in 3 mice the incidence of positive plates was increased.

The results obtained in this study indicate that Salmonella infection in laboratory colonies of mice may be effectively reduced if carriers are isolated and treated with chemotherapy. In our experiments, treatment of infected animals with sulfanilylguanidine resulted in a decrease of Salmonella organisms in stool cultures. In those few instances where chemotherapy is not effective the mice may be sacrificed in order to decrease the sources of infection.

13584

Pigment Observed in Cultures of Hemolytic Streptococci Belonging to Lancefield Group A.*

S. M. WHEELER AND G. E. FOLEY. (Introduced by J. F. Enders.)

From the Department of Preventive Medicine, Harvard Medical School, and House of the Good Samaritan, Boston, Mass.

Pigment production is not often observed in the growth of the hemolytic streptococcus. Durand and Giraud,[1] in a study of 124 cases of human streptococcus infection, reported the isolation of pigmented strains in 11 instances. Olivieri[2] reported the isolation of pigmented hemolytic streptococci from chronic pyelonephritis. Since these studies were made before the development of serological grouping, it is impossible to interpret the observations in terms of the newer classification. More recently, pigment has been observed in strains belonging to Lancefield's Group B, and occasionally to Lancefield's Group D.[3] As far as could be determined from the literature, the phenomenon of pigment production has not been described in strains belonging to Group A.

During an epidemiological study of hemolytic streptococcus infections involving several thousand cultures, pigmented colonies were observed on several occasions in the cultures of 3 patients. A fourth pigmented strain was observed from a case of scarlet fever in an Army camp. The data relating to these strains are summarized in Table I. All strains were classified as to group by the precipitation method of Lancefield,[4] and serological type was determined by

* This work was supported in part by a grant to the House of the Good Samaritan from the Commonwealth Fund.

1 Durand, T., and Giraud, P., *Compt. rendu. Acad. d. sc.*, 1923, **177**, 1333.

2 Olivieri, J., *J. d'urol.*, 1929, **27**, 484.

3 Lancefield, R. C., *J. Exp. Med.*, 1934, **59**, 459.

4 Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571.

TABLE I.
Classification, Frequency and Source of Pigmented Hemolytic Streptococci.

Strain	Source	No. times isolated	Serological group (Lancefield)	Serological type (Griffith)	Non-pigmented strains carried by patients: Previous type	Non-pigmented strains carried by patients: Subsequent type
(1)	throat, scarlet fever	2	A	1-15*	not known	not known
(2)	throat, carrier	2	A	17-23*	28	28
(3)	,, ,,	5	A	3	0	28
(4)	,, ,,	3	A	28	28	0

*Cross-agglutination with type-specific sera.

Note: Recently, 2 more pigmented Group A strains have been recovered from carriers. These were classified respectively as Type 17-23 and Type 28. They were isolated during a streptococcus carrier survey of 700 naval recruits; of whom 176 were positive for Group A hemolytic streptococci.

the Griffith method of slide-agglutination.[5] Cross reactions occurred in these strains only as indicated in Table I.

The results of cultural studies on these strains are summarized in Table II. It seems that the environmental requirements for pigment production on artificial media closely parallel those of the pigmented Lancefield Group B strain included as a control. All media were observed at intervals of 24 hours through 14 days' incubation. Maximum pigment production was observed after 48 hours' incubation on 5.0% horse blood agar, or in buffered dextrose beef infusion broth. Incubation under partial carbon dioxide tension or anaerobic conditions enhanced pigment production in freshly isolated cultures. Partial inhibition was noted in 1.0% horse serum broth. The ability to produce pigment was apparently lost on storage and regained only by prolonged serial culture. Little difference was observed on the various media incubated at 25°C and 37°C.

TABLE II.
Summary of Cultural Results with Various Media.

Strain	Buffered broth	Blood agar	1% serum broth	Loeffler's serum agar	Potato slant	Dextrose serum agar	Sabouraud's agar
(1)	4+	4+	+	—	—	—	—
(2)	4+	4+	+	—	—	—	—
(3)	4+	4+	+	—	—	—	—
(4)	4+	4+	+	—	—	—	—
Control Group A (non pigmented)	—	—	—	—	—	—	—
Control Group B (pigmented)	4+	4+	+	—	—	—	—

4+ = yellowish-brown pigment.
\+ = pale yellow pigment.
— = no pigmentation after 14 days incubation.

[5] Griffith, F., *J. Hyg.*, 1934, **34**, 542.

TABLE III.
Results of Solubility Tests on Ether Extracted Pigment.

Strain	Ether	Chloroform	Carbon disulfide	Alcohol (95%)	Water (dist.)	Normal saline	Sulphuric acid (conc.)
(1)	+	+	+	±	—	—	blue-green color
(2)	+	+	+	±	—	—	"
(3)	+	+	+	±	—	—	"
(4)	+	+	+	±	—	—	"
Control Group A (non-pigmented)	—	—	—	—	—	—	no reaction
Control Group B (pigmented)	+	+	+	±	—	—	blue-green color

+ = soluble.
± = very slightly soluble.
— = insoluble, no color in solvent.

After 48 hours' incubation on 5.0% horse blood agar, the colonies were permeated with a yellowish-brown pigment not unlike that observed in old, dried-out cultures of *Staphylococcus aureus*. The pigment, insoluble in the media used, did not enter the zone of hemolysis on the plates, but seemed to be confined to the colony. The sediment in broth cultures gave much the same appearance. The pigment did not diffuse into the broth, but was confined to the deposit of cells in the butt of the tube.

The results of solubility tests are summarized in Table III. Spectrophotometric analysis indicates that the pigment obtained from the Group A strains is similar to that obtained from the Group B control. (Table IV and Fig. 1.) The pigment could not be extracted from whole cells with the usual organic solvents, but was readily extracted in ether from cultures dried over phosphorus pentoxide and ground in a mortar. The ether extracted pigment was insoluble in normal saline. These results, although preliminary, suggest that the pigmented substance is one of the carotinoids, similar to pigments which are widely distributed in nature.[6] It is not known at this time

TABLE IV.
Summary of Spectrophotometric Analyses.*

Pigment	Solvent	Position of bands (mu.)
Group A Strains	Ether	470-492
	Carbon disulfide	490-508
Group B Strains	Ether	470-492
	Carbon disulfide	490-508

Palmer (1922) has shown by spectroscopic analysis that carotine in ether has a wave-length of 475-490 mu. and 485-519 mu. in carbon disulfide.

*These analyses were made under the supervision of Dr. S. Q. Duntley, Color Measurement Laboratory, Massachusetts Institute of Technology.

[6] Palmer, L. S., *Carotinoids and Related Pigments*, ed. 1, New York, Chem. Cat. Co., 1922, p. 218.

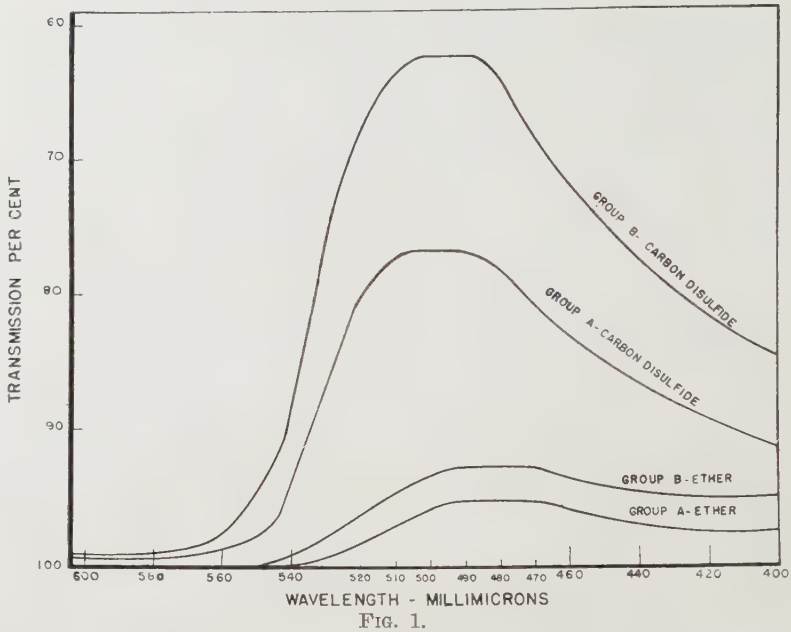


FIG. 1.

Spectrophotometric analyses of pigment extracted from hemolytic streptococci belonging to Group A and Group B.

whether this substance is the same as that recently described by Sevag[7] in a chemical analysis of *"Streptococcus pyogenes,"* in which it was found that the pigmented substance contained a group-specific carbohydrate.

As can be seen in Table I, these pigmented strains were found in throat cultures of only 4 patients over a relatively short period of time. No correlation was observed between pigment and a particular serological type or clinical diagnosis. This does not suggest that pigment production is a characteristic of certain rare strains of Group A hemolytic streptococci. It seems more likely that it is a relatively transient phenomenon occurring only under special environmental conditions.

Failure to obtain maximum pigment production in broth containing 1.0% horse serum, together with the enhancing effect of partial carbon dioxide tension or complete anaerobiasis, suggests a possible relationship of pigment production and the oxidation-reduction potential of the environmental medium. Further experiments are planned using synthetic media of known Eh values to determine the effect of varying oxidation-reduction potentials on pigment production.

[7] Sevag, M. G., *et al., Am. J. M. Sc.*, 1941, **201**, 627.

13585

Effects of Testosterone Propionate in Spayed Female Rats.*

G. L. Laqueur. (Introduced by C. F. Fluhmann.)

From the Department of Obstetrics and Gynecology, Stanford University, School of Medicine, San Francisco, California.

It has been shown[1, 2, 3] that there are well defined differences in the response of female rats to testosterone propionate depending upon the time of the first injection in relation to estrus and diestrus. Since these differences seem to depend on the presence of persisting active corpora lutea in the animals first treated at estrus,[2, 3] the purpose of this study was to determine the effect of castration in order to remove all ovarian factors.

A total of 36 female rats from the Stanford colony was studied. They were divided into 6 groups, each consisting of 6 animals (Table I). They were 107 ± 5 days of age. Their estrous cycles were studied by vaginal smears for 29 days prior to the experiment. Six animals were castrated during estrus, the first injection of testosterone being given at that time. The second group was castrated at the second day of diestrus and testosterone injection was begun at that time. Testosterone propionate suspended in oil was administered subcutaneously in doses of 0.4 cc (4 mg) every second day over a period of 20 days totalling 4 cc or 40 mg. The remaining 4 groups were used as controls. Two were treated exactly as the above except that 0.4 cc of sesame oil was given every second day instead of testosterone. The last 2 groups were given oil only but were not castrated. In all instances the animals were killed 24 hours after the last injection of either testosterone or sesame oil.

The effects of the above treatments are recorded on the accompanying table (Table I). There was a slight difference in the gain of body weights of the rats receiving testosterone, but probably not to a significant degree so that castration apparently inhibited the increase in body weight which has been noted in normal animals.[3] The injection of the male hormone prevented castration atrophy of the

* Supported in part by the Rockefeller Fluid Research Fund of Stanford University, School of Medicine. The testosterone propionate was kindly supplied by the Ciba Pharmaceutical Products, Inc.

1 Wolfe, J. M., and Hamilton, J. B., Proc. Soc. Exp. Biol. and Med., 1937, **37**, 189.

2 Laqueur, G. L., and Fluhmann, C. F., *Endocrinology*, 1942, **30**, 93.

3 Schilling, W., and Laqueur, G. L., submitted for publication.

TABLE I.
Organ Weight Changes in Grams in Spayed Female Rats After Injections of Testosterone Propionate.

Groups	No. of rats	Material injected	cc inj.	Time 1st inj.	Increase body wt	Uter.	Ovar.	Adr.	Thyr.	Liver	Kidn.
Castrates	6	T*	4	E	5.16	.399	—	.035	.014	10.30	1.99
"	6	S†	4	E	4.00	.088	—	.046	.014	10.05	1.78
Normals	6	S	4	E	3.50	.415	.056	.059	.015	8.35	1.41
Castrates	6	T	4	D	4.50	.402	—	.034	.013	9.96	1.97
"	6	S	4	D	3.33	.098	—	.051	.014	10.17	1.82
Normals	6	S	4	D	4.00	.354	.049	.044	.013	8.61	1.51

*T = Testosterone. E = Estrus.
†S = Sesame Oil. D = Diestrus.

uterus regardless whether injected at diestrus or estrus. In all 6 groups no significant difference was observed in the average weights of the thyroid gland and liver while an increase in the weights of the kidneys and a decrease in the weights of the adrenals were observed in the animals given the hormone. The decrease in adrenal weight, however, was directly comparable to the change observed in non-castrates receiving the first injection of testosterone during diestrus.[3]

In castrates treated with testosterone no difference was noted in the histologic appearance of the uteri, mammary glands, and vaginae regardless of the time of the first injection in relation to estrus or diestrus. The morphology of these tissues was exactly the same as that occurring in normal non-castrated females given testosterone at late diestrus.[2] The vaginal epithelium was in a state of mucification, the uterus enlarged and the endometrium proliferated into lace-like folds. The mammary glands exhibited an increase of intertubular fibrous tissue, overgrowth of the ducts, some evidence of secretion but no proliferation of the acini. These findings are distinctly different from those obtained with normal rats receiving the first injection of testosterone at estrus which induces a hypertrophy of corpora lutea.[2]

Summary. In sexually intact female rats genital tissues react differently to testosterone propionate depending upon the time of first injection in relation to estrus and diestrus. In castrates no such difference is apparent. Here the reaction of the genital tissues is the same as those observed when testosterone is first administered at late diestrus to non-castrates.

13586

Cardiac Inhibition of a Cladoceran and the Action of Acetylcholine and Physostigmine.

Vasil Obreshkove.

From Bard College, Columbia University.

It has been held by a group of workers that in physiological processes where nervous impulses are involved and where acetylcholine is believed to act as a transmitter of nervous impulses, physostigmine protects the acetylcholine from the destructive action of choline-esterase. The literature on this subject is fully reviewed by Fraser.[1] For demonstrating the action of these drugs on the heart of a number of crustaceans[2, 3] and on the heart of other invertebrates,[4, 5] perfusion methods have been utilized in bathing the hearts with the chemicals employed. This has made it difficult (by admission of the workers) to obtain data on the separate and combined action of acetylcholine and physostigmine, particularly when considerable time was spent in the dissection and in the preparation of kymographic recordings. It was found in the experimental results recorded here that if the intestine of *Daphnia magna* is touched with a fine glass needle at the bend of the digestive tube where the stomach enters the intestine, the heart immediately ceases beating and the posterior region of the digestive tube commences to exhibit powerful peristaltic waves. The heart in this respect behaves like the inhibition produced after electrical excitation of the vagus nerves in vertebrates. After a certain period, depending on the strength of the mechanical stimulus employed, both the heart and the digestive tube renew their normal activities. The heart comes to rest in the systolic phase of the cardiac cycle and for a period after the escape from inhibition it exhibits high tonicity. Complete cardiac inhibition can also be produced by touching the heart at its posterior dorsal surface. Since the animals utilized in this work were less than 3 mm in body length, and they were transparent, the changes produced can be readily observed under the microscope. In the absence of other data it is assumed that the region stimulated or perhaps other regions in the immediate vicinity, contain nervous elements which conduct inhibitory impulses leading to the arrest of the heart.

1 Fraser, F. R., *Brit. Med. J.*, 1938, **1**, 1293.
2 Welsh, J. H., *J. Exp. Biol.*, 1938, **16**, 198.
3 Welsh, J. H., *Physiol. Zool.*, 1939, **12**, 231.
4 Hamilton, H. L., *J. Cell. and Comp. Physiol.*, 1939, **13**, 91.
5 Prosser, C. L., *Biol. Bull.*, 1940, **78**, 92.

Methods. The method employed in this work in producing complete cardiac inhibition in *Daphnia magna* enabled us to make a study of the effects of acetylcholine and physostigmine on the recovery of the cardiac activity of the animal after escape from inhibition. In order that there may be no doubt as to the specific action of the drugs employed, only animals which showed very slow cardiac recovery after the arrest of the heart were chosen for the treatment with chemical substances and for the analysis presented here. The animals were subjected to experimentation soon after the release of the first clutch of young. The method of rearing cladocera in the laboratory and the methods employed in the selection of animals which were in the same stage of development have been described elsewhere.[6] The technic employed in administering acetylcholine and physostigmine was the same as that employed for the study of the action of these drugs on the intestine of *Daphnia magna.*[7] The animals were subjected to experimentation separately; a single individual in each case was transferred to a micro culture slide for the treatment with specific chemical substance employed and for observations under the microscope. The solutions were made daily and the acetylcholine was adjusted in each case to pH 5.7. The rate of the heart contractions per minute was recorded by dots on a piece of paper. This method was found accurate for counting heart contractions of about and below 150 per minute but above that it was found more difficult to synchronize the recording of dots with the heart contractions. The error in counting extremely rapid heart beats is not of importance, for the more rapid cardiac activity simply furnishes more unquestionable evidence of the action of the drugs in inducing the specific changes.

Results. Table I presents the results obtained when the heart of *Daphnia magna* was arrested by producing inhibitory impulses with a mechanical stimulus applied over the intestine and then treating the animal with acetylcholine. In this table are given the "normal rate" of the heart beat, the period of heart arrest in each case, the frequency of beat for a period of 2 minutes after the escape from cardiac inhibition as well as after the addition of acetylcholine 10^{-7}. In each case, without a single exception, the heart after the escape from inhibition and before the treatment with acetylcholine showed grouped beats which were feeble in character and irregular in amplitude. This condition persisted for several minutes. After the addition of acetylcholine, however, there was observed a sudden increase in the

[6] Obreshkove, V., *Physiol. Zool.*, 1930, **3**, 271.

[7] Obreshkove, V., *Biol. Bull.*, 1941, **81**, 105.

frequency of the heart beat and the cardiac activity became immediately more powerful and regularly rhythmic in character. The drug became effective in each case in less than 20 seconds.

The period of heart arrest is shown in these experiments to vary from 2.5 to 20.7 minutes (column 2). The rate of heart beat during the first minute after escape from inhibition and before the administration of acetylcholine was found to be on the average 57 beats per minute and only slight change in this rate was observed during the following 60 seconds (column 3) and in the course of several minutes later. In the first few seconds after the addition of acetylcholine, however, the rate became rapid (column 4) and in some cases the frequency of beats during the first minute equaled and exceeded the normal rate. Similar observations were recorded with acetylcholine

TABLE I.

Action of Acetylcholine on Recovery of Heart of *Daphnia magna* (First Brood Mothers) from Cardiac Inhibition.

Rate of heart beat expressed in beats per minute.

Normal heart rate	Period of heart arrest min.	Heart beat after recovery		Heart beat in Acetylcholine 10^{-7}	
		Rate	Min. after recovery	Rate	Min. after recovery
226	17.2	18	1	122	1
		36	2	144	2
234	2.3	24	1	246	1
		30	2	244	2
238	20.7	36	1	98	1
		44	2	104	2
250	16.6	72	1	170	1
		130	2	180	2
260	19.3	80	1	122	1
		80	2	130	2
236	2.1	54	1	102	1
		52	2	100	2
246	6.1	86	1	250	1
		184	2	250	2
246	5.3	36	1	84	1
		32	2	86	2
228	14.3	84	1	204	1
		168	2	200	2
238	2.5	80	1	104	1
		80	2	106	2
Avg 240.2		57.0	1	150	1
		83.6	2	154.4	2

10^{-5} and 10^{-9}. The changes occurring in each case after the application of the drug were definite in character and were of sufficient magnitude to preclude any conclusions pertaining to the action of acetylcholine on the cardiac activity of this animal.

Physostigmine. If the acetylcholine is the chemical mediator between nerve terminations and the cardiac muscle in *Daphnia magna,* it should be possible to demonstrate this by the appropriate administration of physostigmine, for the protective action of physostigmine on acetylcholine might lead to increased heart activity. When individuals were eserinized for a period which varied from 5 to 15 minutes and after this, cardiac inhibition was produced in the usual way mechanically, it was observed that immediately after escape from inhibition, the cardiac rhythm became regular and there was a quick reëstablishment of the normal rate of heart beat, without treating the animals with acetylcholine. The action of physostigmine 10^{-4} is shown in Table II. In addition to this a range of other concentrations were employed which varied in strength from 10^{-3} to 10^{-7} and the observations recorded were similar to those shown in Table II. This rapid recovery of the cardiac activity of eserinized individuals is in marked contrast with the situation found in animals which were not (previous to the arrest of the heart) treated with physostigmine (Table I, column 3).

In the course of the eserinization of the animals there was also observed an acceleration of the heart beat but the action of physostigmine was not immediate. The rate of heart beat under the influence of physostigmine 10^{-4}, before cardiac inhibition presented in Table II does not represent the maximum cardiac activity under the influence of this drug.

Discussion. Repeated endeavors by staining methods to demonstrate clearly the nerve supply to the heart of *Daphnia magna* were beset by many difficulties. We are in possession, however, of cer-

TABLE II.

Action of Physostigmine on Recovery of Heart of *Daphnia magna* (First Brood Mothers) from Cardiac Inhibition.

Rate of heart beat expressed in beats per minute.

Normal heart rate	Treatment with Physostigmine 10^{-4}, min.	Rate of beat before heart arrest	Period of heart arrest, min.	Heart rate, 1 min. after recovery
260	10	300	6.7	270
240	10	264	5.7	246
240	15	254	1.7	256
236	5	242	2.2	152
246	10	252	1.7	246
Avg 244.4		262		234

tain facts pertaining to the nervous system of a number of larger Crustacea. Carlson[8] has demonstrated a pair of nerves arising from the thoracic ganglion of *Palinurus;* one of these when stimulated has been found to inhibit the heart and the other to be acceleratory in function. The functional activity of extrinsic nerves to the heart has been abundantly confirmed also for a number of other Crustacea by this same worker and the nervous elements within the heart have been described and illustrated by Alexandrowicz.[9] Although there is nothing in Crustacea, insofar as we know, which corresponds morphologically to the autonomic nervous system in vertebrates, it is evident from the observations presented here that the intestine and the heart of *Daphnia magna* are subject to acceleratory and inhibitory nervous influences. In the mammals the parasympathetic transmitter is believed to be identical with acetylcholine and causes cardiac inhibition. The actions of this drug and of physostigmine on the heart of *Daphnia magna* are the direct opposite of their effects in mammals but are in accord with those reported for a number of invertebrates.[10] Artemov and Mitropolitanskaja[11] have demonstrated the presence in whole *Daphnia* of an acetylcholine-like substance. As yet, however, no one has undertaken to demonstrate the presence or absence of choline esterase in this group of animals. The questions of how acetylcholine, if present in *Daphnia,* is bound in the tissues and how it is protected, and the question of the mechanisms involved in the specific effects of acetylcholine and physostigmine in invertebrates as those contrasted in vertebrates must wait for further investigations before they are answered.

Summary. The heart of *Daphnia magna* can be arrested by applying a mechanical stimulus to a region in the digestive tube where the stomach enters the intestine. This induced inhibition and likewise the recovery after inhibition, has enabled us to make a study of the action of acetylcholine and physostigmine on the possible control and coördination of the heart beat. If the animal is treated with acetylcholine when the heart beat, after escape from inhibition, is still feeble, slow and irregular, there is observed an immediate acceleration in the heart beat and the cardiac activity becomes more powerful and rhythmic in character. Animals treated with physostigmine previous to the arrest of the heart, show complete restoration of the regular beat soon after the escape from inhibition.

8 Carlson, A. J., *Am. J. Physiol.*, 1905, **15**, 127.

9 Alexandrowicz, J. S., *Quart. J. Micro. Sci.*, 1932, **75**, 18.

10 Walsh, J. H., *Physiol. Zool.*, 1939, **12**, 231.

11 Artemov, N. M., and Mitropolitanskaja, R. L., *Bull. de Biol. ed de Méd. Exp. U.R.S.S.*, 1938, **5**, 378.

13587

Influence of Fasting on Retention and Conjugation of Sulfanilamide in Rabbits.

E. F. Stohlman and M. I. Smith.

From the Division of Chemotherapy, National Institute of Health, Bethesda, Md.

It has been shown[1] that rats maintained on a low protein diet exhibited higher drug blood levels when treated with sulfanilamide than rats treated similarly but receiving adequate protein in their diet. In like manner therapeutic tests with sulfanilamide in mice with streptococcus infection showed the drug to be more effective in animals maintained on a low protein diet, and the degree of effectiveness appeared to be related to the blood concentration of the drug.[2] Since the food intake is likely to be much curtailed in animals restricted to low protein diets, it seemed desirable to ascertain the fate of sulfanilamide under fasting conditions.

Rabbits were selected for these experiments for the following reasons: (1) It was desirable to have an animal large enough to be suitable for frequent bleedings; (2) the rabbit is suitable for frequent catheterization so that the excretion curve could be followed; and (3) normally, sulfanilamide is acetylated in the rabbit to a very high extent and with considerable uniformity.

Procedure. The rabbits were catheterized and 0.5 g per kilo sulfanilamide suspended in about 25 cc 5% gum acacia was administered by stomach tube. The drug was washed in with about 100 cc water to insure moderate diuresis. Small blood samples were taken from the ear vein for sulfanilamide determinations at intervals of 1, 2, 4, 6, and 24 hours or longer if necessary. Urine was collected at 24-hour intervals up to 72 hours when necessary to determine the excretion rate of the drug. The method of Bratton and Marshall[3] was used for the colorimetric determination of free and total sulfanilamide.* The animals were on a diet of oats and cabbage for at least a week prior to the fasting period, during which time water alone was permitted.

Results. In the normal non-fasting rabbit the peak blood con-

[1] Smith, M. I., Lillie, R. D., and Stohlman, E. F., *Public Health Rep.*, 1941, **56**, 24.

[2] Rosenthal, S. M., *Public Health Rep.*, 1941, **56**, 188.

[3] Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

* In some of the experiments the method of A. S. Giordano and M. C. Prestrud (*Am. J. Clin. Path.*, 1940, **10**, 88) was used.

TABLE I.
Absorption, Conjugation, and Excretion of Sulfanilamide in Rabbits Under Diverse Experimental Conditions. Dose—0.5 g per kg orally.

Experimental condition	No.	Maximum blood level		Urinary excretion in 24 hours		
		Mg%	Conjugation %	% of dose	Conjugation %	Urine, cc/hr
Normal	2	41	79	75	90	9.8
"	3	45	88	89	91	8.5
"	25	50	59	90	83	11.8
"	55	38	79	70	85	10.4
Avg		44	76	81	87	10.1
Fasting. 3 days	16	54	60	75	70	8.2
" 7 "	17	92	54	70	44	13.0
" 9 "	18	45	27	65	53	8.7
" 14 "	19	55	33	50	45	8.0
Avg		61	43	65	53	9.5
Carbon tetrachloride	42	32	51	96	81	13.0
" "	43	41	46	71	75	6.3
" "	44	40	63	95	73	11.1
Avg		37	53	87	76	10.1
Acidosis						
Oats diet, 9 days	23	41	57	72	52	5.3
" " 26 "	26	31	69	84	79	10.9
" " 28 "	27	30	57	46	49	7.2
NH_4Cl, 1 g per kg orally	33	23	82	60	89	9.6
" 0.5 " " " "	34	29	68	70	88	12.6
NaH_2PO_4, 1 " " " "	64	30	30	51	81	8.3
" 1 " " " "	65	25	28	70	80	7.7
" 1 " " " i.v.	66	20	51	79	78	18.5
" 1 " " " "	68	27	55	61	81	11.2
Avg		28	55	66	75	10.1

centration of sulfanilamide under these conditions was reached in 2 to 4 hours, with an average of 76% acetylation. The elimination of sulfanilamide in the urine during the first 24 hours was on an average 81% of the dose administered, and 87% of this was acetylated. The urine output, which varied in these experiments from 8.5 to 11.8 cc per hour, seemed to bear no relationship either to the excretion or the acetylation of the drug. There was usually little or no sulfanilamide in the blood at the end of 24 hours. This is shown in the first part of Table I.

The data given in Table II show generally higher blood levels, decreased rate of conjugation and an increasing rate of retention of the drug with progressive fasting. Thus after a fast of 18 hours the free sulfanilamide was only 12% of the total in the blood 6 hours

TABLE II.
Effect of Fasting on Blood Concentration and Conjugation of Sulfanilamide in Rabbits. Dose 0.5 g per kg orally.

Hrs after administration	Blood sulfanilamide concentration T = total, mg%; F = free, % of total									
	18 hrs		3 days		7 days		9 days		14 days	
	T	F	T	F	T	F	T	F	T	F
1	19	80	17	68	50	67	30	73	50	67
2	44	49	36	40	60	74	50	64	55	79
4	45	21	42	55	92	46	43	81	44	77
6	43	12	54	40	80	72	45	73	43	80
24	4	0	22	1	45	74	27	74	33	76
48	0	0	7	0	17	59	10	69	18	72
72	0	0	0	0	5	58	10	45	12	63

after administration of 0.5 gm per kilo; while after a 14-day fast, it was 80% of the total. After a 14-day fast, the blood level 24 hours after the administration of the drug was 33 mg %, 76% of which was free; while after an 18-hour fast, the blood level at this time was only 4 mg %, and all of this was conjugated. In an animal fasting several days there may still be considerable sulfanilamide in the blood as long as 72 hours after its administration. In like manner the excretion of the drug is nearly complete in 24 hours in the normal animal but may not be more than 50% of the dose administered in the fasting animal at this time (Part 2, Table I).

The present experiments confirm and extend the earlier observations.[1] A satisfactory explanation for these findings is not at hand, though several possibilities suggest themselves. The possibility of impaired hepatic or renal function in prolonged fasting cannot be excluded, though this does not seem probable. A direct approach to this was made by administering sulfanilamide in the usual manner to rabbits that had been previously treated for a week with daily doses of 1 cc per kilo of carbon tetrachloride. The fate of sulfanilamide in such animals was not materially different from normal, as may be seen in Part 3 of Table I, except possibly for some slight reduction in the rate of conjugation. This is consistent with the reported site of sulfanilamide conjugation in the liver of the rabbit.[4, 5] The urine output in our fasting animals during sulfanilamide treatment was within normal limits.

The possibility of a state of acidosis developing in the course of fasting was also considered. With this in view the fate of sulfanilamide was examined in a series of rabbits on an exclusive oats diet, which is acid-producing, and also in animals receiving ammonium

[4] Stewart, J. D., Rourke, G. M., and Allen, J. G., *Surgery*, 1939, **5**, 232.

[5] Van Winkle, W., and Cutting, W. C., *J. Pharm. and Exp. Ther.*, 1940, **69**, 40.

chloride or sodium acid phosphate simultaneously with the sulfanilamide. These experiments indeed showed a reduced rate of conjugation in the blood and a somewhat decreased rate of elimination of the drug as compared with controls, as shown in Part 4 of Table I. The effects, however, were not as pronounced as in fasting. The lower peak in the blood under these conditions as compared with controls suggests a reduction in the rate of absorption of the drug. Obviously more work is needed to elucidate the effects of fasting or low protein diet on the fate of sulfanilamide in the body.

Summary. Prolonged fasting in the rabbit favors the absorption and retention of sulfanilamide, producing higher blood levels of the drug and over a longer period of time as compared to normally fed animals. This also favors reduction of acetylation of the drug. An acid producing diet or the administration of drugs favoring a state of acidosis appears to have an effect on the fate of sulfanilamide similar to that of fasting.

13588

Action of Phlorizin on Acid Phosphatase Activity and on Glucose Phosphorylation of Kidney Cortex Extracts.*

LYLE VIBERT BECK. (Introduced by J. F. McClendon.)

From the Department of Physiology, Hahnemann Medical College, Philadelphia, Pa., and the Marine Biological Laboratory, Woods Hole, Mass.

1. *Phosphatase Experiments.* Several authors[1, 2, 3] have reported that phlorizin does not appreciably affect the phosphatase activity of kidney extracts. In all cases their findings are apparently concerned with the alkaline phosphatase, since the determinations were made within the pH range of 7.6 to 9.2. It was thought of interest to determine the effects of phlorizin on the acid phosphatase activities of kidney cortex and intestinal mucosa extracts.

The filtered extracts were prepared as described by Kay,[4] brought

* The author is indebted to Dr. C. H. Fiske for animal adenylic acid, and to Miss Ethel Shiels and Dr. Marshall Smith for many glass electrode pH determinations.

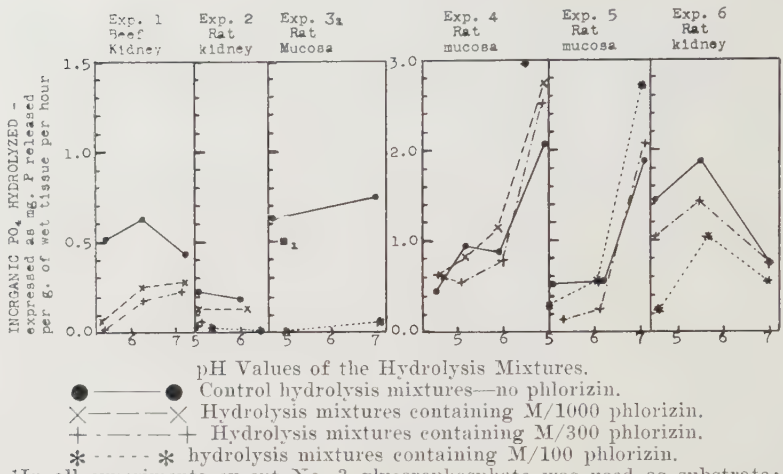
1 Lambrechts, A., *Arch. intern. physiol.*, 1937, **44** Suppl., 136.

2 Walker, A. M., and Hudson, C. L., *Am. J. Physiol.*, 1937, **118**, 130.

3 Kritzler, R. A., and Gutman, A. B., *Am. J. Physiol.*, 1941, **134**, 94.

4 Kay, H. D., *Biochem. J.*, 1928, **22**, 855.

FIGURE I - PHOSPHATASE ACTIVITIES IN ACID pH RANGE



pH Values of the Hydrolysis Mixtures.
●———● Control hydrolysis mixtures—no phlorizin.
×— — —× Hydrolysis mixtures containing M/1000 phlorizin.
+ - - - - + Hydrolysis mixtures containing M/300 phlorizin.
✻ - - - - - ✻ hydrolysis mixtures containing M/100 phlorizin.

[1]In all experiments except No. 3 glycerophosphate was used as substrate; in this experiment hexosediphosphate served as substrate. The square symbol in Exp. 7 represents a control value obtained using glycerophosphate.

to pH 5.2 by addition of 1 part in 7 of veronal-acetate buffer, to preserve the acid phosphatase, and kept on ice until used. Determinations of phosphatase activity were carried out at 37°C as described by Belfanti and coworkers.[5] The results are shown in Fig. 1.

At pH about 5 the rate at which inorganic phosphate is formed from glycerophosphate in the presence of kidney cortex extracts is markedly decreased by m/100 phlorizin, and in some instances is appreciably decreased by m/300 and even m/1000 phlorizin. With increase in pH the inhibition becomes less, until at and beyond pH 7 (up to pH 9) even m/100 phlorizin has practically no inhibitory action on the phosphatase activity.

When intestinal mucosa extracts were employed marked inhibition was secured only in Exp. 3, in which hexosediphosphate was employed as substrate. In 2 other experiments, using glycerophosphate as substrate, the results at pH below 7 were irregular, and at pH about 7 phlorizin actually produced an acceleration of the rate of disappearance of inorganic phosphate. In the alkaline pH range (7 to 9) the values for control tubes and for phlorizin-containing tubes were practically identical.

II. *Glucose Phosphorylation Experiments.* Kalckar[6] has succeeded in demonstrating phosphorylation of glucose in the presence

[5] Belfanti, S., Contardi, A., and Ercoli, A., *Biochem. J.*, 1935, **29**, 517.

[6] Kalckar, H., *Enzymologia*, 1937, **2**, 47.

of rabbit kidney cortex extracts. He has reported that this phosphorylation is strongly inhibited by phlorizin (about 60% by m/600) and has revived Lundsgaard's hypothesis[7] that phlorizin prevents glucose reabsorption by inhibiting glucose phosphorylation within these cells. In view of this hypothesis and Kalckar's observations it was thought of interest to determine the lowest concentration of phlorizin capable of producing practically complete inhibition of glucose phosphorylation by kidney cortex extracts, since we have estimates[1, 8] of the lowest *in vivo* concentration of phlorizin required to completely inhibit glucose reabsorption by the kidney (see below). Experiments similar to those of Kalckar have therefore been performed.

Ground up rabbit kidney cortex-sand brei was mixed with m/15 Na_2HPO_4 (1.5 cc for each gram of tissue), vigorously shaken for 10 minutes, and the clear supernatant fluid obtained on centrifuging used for the experiment. Two arm tubes were used. 1.0 cc of enzyme extract was placed in one arm, 0.5 cc of fluid containing other substances in the other. After temperature equilibration in a water bath at room temperature (about 22°C), the contents of the two arms were mixed, the time noted, and an aliquot blown into 10% trichloracetic acid for determination of original inorganic phosphate. At desired intervals other aliquots were taken. About the middle of the 1- or 2-hour experimental period a glass electrode pH determination was made on the phosphorylation mixture. Throughout the experimental period the tubes were rocked back and forth in the water bath to assure aeration of the mixture.

NaF was used in n/30 final concentration. When present the concentration of adenylic acid in the phosphorylation mixture was 5 mg %, that of sodium succinate n/150 and that of hexose 1.5%. By analysis that of inorganic phosphate was about 50 mg %.

The results are shown in Table I. The lowest concentration of phlorizin which will still produce practically complete inhibition of glucose phosphorylation is about m/333. It is interesting to note that very low concentrations of phlorizin (m/3333 and m/10,000) increase the rate of disappearance of inorganic phosphate.

Discussion. Lundsgaard[7] found that the minimum concentration of phlorizin required to markedly inhibit hexose phosphorylation brought about by muscle brei and dried yeast is about m/50 to m/100, and suggested that phlorizin may inhibit glucose reabsorption in the kidney by interfering with its phosphorylation. Later[8] he

[7] Lundsgaard, E., *Biochem. Z.*, 1933, **264**, 209.

[8] Lundsgaard, E., *Skand. Arch. f. Physiol.*, 1935, **72**, 265.

TABLE I.

Mg of Inorganic Phosphate P Disappearing, per Gram of Wet Kidney Cortex Tissue per Hour, in Presence and Absence of Adenylic Acid, Glucose, and Phlorizin.

Exp. No.	A.A.* present?	Sugar used	Conc. of phlorizin	pH of exper. mixture	Inorg. P disappearing: As mg	Inorg. P disappearing: As % of avg control value
I	Yes	0	0	—	0.01	1
		Glucose	0	7.17	1.46 Avg.	
				7.23	1.14 1.18	100
				7.17	0.93	
			m/333	7.29	—0.02	— 2
			m/1000	7.39	0.00	0
II	Yes	Glucose	0	6.80	1.12 Avg,	
				7.20	1.17 1.34	100
				7.30	1.62	
			m/500	7.25	0.40	30
			m/1000	7.12	1.69	126
			m/3333	7.22	1.71	128
			m/10,000	7.30	1.91	147
III	No	0	0	7.20	0.34	35
	Yes	0	0	7.41	0.55	57
		Glucose	0	7.31	0.88 Avg,	
				7.50	1.06 0.97	100
			m/333	7.42	0.34	35
			m/1000	7.34	0.90	93
			m/3333	7.32	1.10	113
			m/10,000	7.21	1.21	125
IV	No	0	0	6.97	0.10	
	Yes	0	0	7.31	0.33	
	Yes	Glucose	0	6.98	0.96	100
			m/333	7.08	0.02	2
		Fructose	0	6.89	1.89	100
			m/333	—	0.05	3
		Galactose	0	7.14	0.72	100
			m/333	7.20	—0.10	— 14

*A.A. equals 5 mg% adenylic acid plus n/150 Na succinate present or not present in the phosphorylation mixture.

estimated that the minimum *in vivo* concentration of phlorizin required to practically completely inhibit glucose reabsorption by the dog kidney is of the order of 0.5-1.0 mg per g of kidney tissue (0.75 mg = m/630), and concluded that the phosphorylation hypothesis was not entirely satisfactory. By another method, Lambrechts[1] arrived at a value of m/1000 phlorizin or less, as compared with Lundsgaard's value of about m/630. However, Lambrechts also found that 2 highly colored and strongly glycosuric phlorizin deriva-

tives are preferentially absorbed and retained in the proximal tubule cells, the exclusive site of active glucose reabsorption.[2, 9] He concluded that the concentration of phlorizin at its point of action within the kidney cells might be several times as great as m/1000.

The observations of Kalckar and the data presented here demonstrate that the hexokinase of rabbit kidney cortex is far more sensitive to phlorizin poisoning than the hexokinases studied by Lundsgaard. These various considerations indicate that (X), the minimum concentration of phlorizin required to completely inhibit glucose reabsorption *in vivo,* and (Y), the minimum concentration required to completely inhibit glucose phosphorylation *in vitro,* are close enough together to satisfy Lundsgaard's phosphorylation hypothesis of glucose reabsorption.

Although the phosphatase activity of rat kidney cortex extracts is quite sensitive to phlorizin at pH about 5, it shows practically no sensitivity in the intracellular pH zone (about 6.9).[10] It therefore seems unlikely that interference with glucose reabsorption by phlorizin can be due to any appreciable extent to inhibition of dephosphorylation.

13589

Effect of Vitamin D on Prothrombin Deficiency in the Rat.

F. S. GRODINS AND A. C. IVY.

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

It has been reported[1] that the prothrombin deficiency produced in rats by a diet containing 20% mineral oil was corrected by vitamin K, and definitely improved by vitamin D administration. Previously, Smith and coworkers[2] had reported that vitamin D failed to improve the prothrombin deficiency in a biliary fistula dog, and Greaves[3] stated that massive doses of D subcutaneously were without benefit in biliary fistula rats. However, vitamin D has been shown to

9 Walker, A. M., Bott, P. A., Oliver, J., and MacDowell, M. C., *Am. J. Physiol.*, 1941, **133**, 480.

10 Chambers, R., *Bull. Nat. Research Council*, No. 69, 1929, Washington, D.C.

1 Eliot, Isaacs, and Ivy, PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 240.

2 Smith, Warner, Brinkhous, and Seegers, *J. Exp. Med.*, 1938, **67**, 911.

3 Greaves, *Am. J. Physiol.*, 1939, **125**, 423.

be of value in treating the bleeding tendency in clinical jaundice.[4] In view of these conflicting reports, we have further investigated the effect of vitamin D on prothrombin deficiency in the rat.

A prothrombin deficiency was produced in 36 normal rats by means of the diet described by Eliot, Isaacs and Ivy[1] (Table I, Group 1). Quick's method[5] for the estimation of prothrombin was used. After 29-52 days on the diet, the prothrombin time was prolonged from an average normal of 17.6 seconds to a mean of 59.7 seconds.

Eighteen rats whose prothrombin times had been increased by the diet from a normal average of 18.0 seconds to 53.4 seconds were given 500 units of vitamin D* subcutaneously. One week later the average prothrombin time of the group was 55.4 seconds (Table I, Group 2). There is no significant difference between the average values before and after treatment with vitamin D. Individually, 5 of the 18 appeared significantly improved. Ten of these rats were then given an additional dose of 1000 units of vitamin D subcutaneously (Table I, Group 3). One week later their average prothrombin time was 71.8 seconds compared to 41.2 seconds before the second dose was given. Again in this group, vitamin D failed to improve the prothrombin deficiency.

TABLE I.

		Prothrombin time—seconds															
								After Vitamin D									
								1 week (500 U)			3-5 days (1000 U)			1 week (1000 U)		
Group No.	No. rats	Controls			After diet												
		M	R	σ	M	R	σ	M	R	σ	M	R	σ	M	R	σ
1	36	17.6	13.6 22.4	.017	59.7	18.8 178.1	5.5									
2	18	18.0	14.9 22.0	.46	53.4	34.4 105.1	4.7	55.4	26.9 103.3	5.4						
3	10	17.7	16.1 22.2	.62	48.3	35.9 82.4		41.2	27.7 60.5	4.13				71.8	31.2 156.6	14.6
4	15	18.0	14.7 19.5	.37	71.6	39.3 178.1	9.9				50.8	18.3 **98.8**	6.5	50.2	29.6 **101.0**	5.6

M = Mean.
R = Range.
σ = Standard Error of Mean.

[4] Gray and Ivy, *Am. J. Dig. Dis.*, 1935, **2**, 368; McNealy, Shapiro, and Melnick, *Surg., Gynec., and Obst.*, 1935, **60**, 785.

[5] Quick, *J. A. M. A.*, 1938, **110**, 1659.

* Abbott Viosterol.

Since these results disagreed with those previously reported,[1] a third group of 15 deficient animals was given 1000 units of D† subcutaneously and the prothrombin time was determined 3-5 days and 1 week later (Table I, Group 4). In this group, the mean prothrombin values before and after treatment show a questionably significant difference. If the extreme variates are omitted in calculating the mean in column 6, the differences become insignificant. Individually, 7 of the 15 were definitely improved, 4 were more deficient after receiving the vitamin than before, 3 were essentially unchanged, and 1 died before a second determination was made.

Comment. These results fail to show that vitamin D has any definitely beneficial effect on the prothrombin deficiency produced by dietary means in the rat. Although certain individual cases showed improvement, the average results of the group show no striking effect. This does not agree with the interpretation of results previously reported.[1] The reason for this difference is not immediately apparent; however, the range of variation in the deficient rats is so great that it is difficult to evaluate any procedure unless a large series is used. This may explain the difference. The present results agree with those of Smith and coworkers[2] and of Greaves.[3] However, they fail to explain the beneficial effects of viosterol in clinical jaundice.

13590

Use of Avidin in Studies on Biotin Requirement of Microorganisms.*

MAURICE LANDY, DOROTHY M. DICKEN, MARGARET M. BICKING AND WINIFRED R. MITCHELL. (Introduced by Paul György.)

From the Research Laboratories of the S.M.A. Corporation, Chagrin Falls, Ohio.

The recent discovery of anti-vitamins suggests a new principle in nutritional pathology, with far-reaching applications in the study of bacterial metabolism. Avidin,[1, 2] the first anti-vitamin to be iso-

† Meade-Johnson Special Concentrate.

* Presented at the forty-third general meeting of the Society of American Bacteriologists, Baltimore, Maryland, December 29, 1941.

1 Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **136**, 801.

2 Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1941, **140**, 535.

lated,[3] belongs to a group[4] of nutritionally important substances which may be as potentially significant as the vitamins themselves, in that they act as governors for the biological processes which are catalyzed by the vitamins. The anti-vitamin, avidin (anti-biotin[5]), is a protein constituent of unheated egg white, which possesses the property of combining readily and specifically with biotin to form a stable complex—a complex in which the biotin is rendered unavailable for utilization by yeast cells,[2] by animals[6, 7] and, as we propose to demonstrate, by certain bacteria.

This report constitutes a study of the effect of avidin on various bacterial species. We anticipated a correlation between the biotin requirement of an organism and its subsequent growth inhibition by avidin. If such a correlation were found to exist, it would obviously follow that the biotin requirement of a given organism might be predicted by its reaction to avidin. It is necessary to point out that our tests were only qualitative and, due to circumstances beyond our control, fairly crude. Two properties peculiar to avidin make its sterilization difficult: (1) it is heat labile; *i. e.*, solutions are inactivated rapidly by steaming or autoclaving, and (2) it is, for the most part, retained by bacterial filters.† It was necessary, therefore, to employ fresh sterile egg white for many experiments, although a number have been carried out with purified avidin preparations yielding identical results.

The general method of procedure was as follows: Serial dilutions of avidin concentrate or egg white were added to duplicate sets of tubes of synthetic or infusion medium containing known amounts of biotin. The avidin in one set of dilutions was inactivated by autoclaving for 15 minutes while the other set was not heated. Both series of tubes were then inoculated with the test organism. The effect of avidin on the test organism, as indicated by the degree of growth inhibition produced, was determined by a comparison of growth in the heated and unheated avidin dilutions. The addition of biotin to those dilutions of avidin which brought about complete inhibition of growth resulted in growth of the organism upon reincubation.

---

[3] Pennington, D., Snell, E. E., and Eakin, R. E., *J. Am. Chem. Soc.*, 1942, **64**, 469.

[4] Woolley, D. W., *J. Biol. Chem.*, 1941, **141**, 997.

[5] Woolley, D. W., and Longsworth, L. G., *J. Biol. Chem.*, 1942, **142**, 285.

[6] Gyorgy, P., Rose, C. S., Eakin, R. E., Snell, E. E., and Williams, R. J., *Science*, 1941, **93**, 477.

[7] Gyorgy, P., and Rose, C. S., *Science*, 1941, **94**, 261.

† Unpublished data.

It was our practice to add sufficient avidin to any given culture to neutralize the biotin present and still insure an excess of avidin. It is important to emphasize that this investigation was limited to a study of the effect of avidin in such concentrations. That higher concentrations of avidin would yield entirely different results is, of course, a possibility. Serial dilutions of avidin were carried out in such a manner that complete inhibition, partial inhibition and complete growth of a susceptible organism were obtained.

Early in the investigation, it occurred to us that certain effects which we observed might be due to lysozyme present in egg white or avidin concentrate. That this was not the case was shown by a number of experiments in which it was found (1) that purified, highly potent lysozyme preparations had no effect on organisms which were markedly susceptible to avidin, *viz.*, the saccharomyces, the clostridia, and the lactobacilli, (2) that our avidin preparations had no effect on the usual lysozyme-assay with *Micrococcus leisodykticus*. In general, we found lysozyme and avidin to have separate, definite and apparently unrelated biological activities. In all experiments in which egg white or avidin inhibited growth of an organism, our final criterion of the specificity of such inhibition was its reversal by crystalline biotin.

Lactobacillus arabinosus 17-5,[8] *Streptococcus lactis* R,[9] *Staphylococcus aureus* X-3,[10] *Clostridium butylicum*,[11] and *Clostridium acetobutylicum*[12] have been shown to require biotin for growth. As will be observed in Table I, growth of the above organisms was, in every case, completely inhibited by avidin, which inhibition could, in turn, be reversed by the addition of biotin. On the other hand, the growth of such organisms as *Eberthella typhi, Proteus vulgaris, Escherichia coli, Aerobacter aerogenes,* and *Serratia marcescens* which grow in simple media and synthesize biotin[13] was unaffected by avidin. Since avidin inhibits the growth of the above organisms which require biotin, and has no effect upon those which do not require biotin, this logical correlation suggests the use of avidin as an agent in the determination of the biotin requirement of microörganisms.

On the basis of the evidence presented we feel justified in predicting that those organisms given in Table I which are inhibited by

8 Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

9 Snell, E. E., and Mitchell, H. K., *Proc. Nat. Acad. Sc.*, 1941, **27**, 1.

10 Porter, J. R., and Pelczar, M. J., *J. Bact.*, 1941, **41**, 173.

11 Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1939, **61**, 3594.

12 Oxford, A. E., Lampen, J. O., and Peterson, W. H., *Biochem. J.*, 1940, **34**, 1588.

13 Landy, M., and Dicken, D. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 449.

TABLE I.
Effect of Avidin on the Growth of Bacteria.

Organism	Medium	Incubation °C	Incubation Hrs	Degree of Inhibition	Biotin Reversal of Inhibition
Lactobacillus casei ϵ	Synthetic[14]	37	72	Complete	+
Lactobacillus arabinosus (17-5)	" 8	30	48	"	+
Lactobacillus acidophilus	" 14	37	72	"	+
Streptococcus lactis (R)	" 9	30	72	"	+
Staphylococcus aureus (X-3)	Heart Infusion Broth	37	48	"	+
Staphylococcus aureus (Y)	" " "	"	"	"	+
Diplococcus pneumoniæ (Mellon II)	" " "	"	"	"	+
Corynebacterium diphtheriæ (Park 8)	" " "	"	"	None	
Clostridium welchii (SR-12)	Thioglycollate	37	72	Complete	+
Clostridium welchii (Spray)	"	"	"	"	+
Clostridium botulinum (201-B)	"	"	"	"	+
Clostridium histolyticum (Spray)	"	"	"	"	+
Clostridium chauvei	"	"	"	"	+
Clostridium sporogenes	"	"	"	"	+
Clostridium butylicum (2-D)	Synthetic[11]	"	"	"	+
Clostridium acetobutylicum (A-211)	" 15	"	"	"	+
Klebsiella pneumoniæ	Heart Infusion Broth	37	48	None	
Eberthella typhi	" " "	"	"	"	
Salmonella enteritidis	" " "	"	"	"	
Serratia marcescens	" " "	"	"	"	
Bacillus subtilis	" " "	"	"	"	
Bacillus brevis	" " "	"	"	Complete	+
Bacillus anthracis	" " "	"	"	None	
Escherichia coli	" " "	"	"	"	
Alkaligenes fæcalis	" " "	"	"	"	
Aerobacter aerogenes	" " "	"	"	"	
Proteus vulgaris	" " "	"	"	"	
Proteus morganii	" " "	"	"	"	
Shigella paradysenteriæ	" " "	"	"	"	
Vibrio comma	" " "	"	"	"	

avidin (inhibition reversed by biotin) require biotin as a growth-essential. In partial confirmation of this prediction we have since demonstrated that *Lactobacillus casei* ϵ can be cultured in a medium of essentially-known composition only when biotin is supplied.[14]

Summary. A study was made of the effect of avidin on the growth of a number of bacterial species. A correlation was found between the biotin requirement of an organism and its growth-inhibition by avidin. In general, those organisms which have been found to require biotin as a growth-essential were inhibited by avidin, while those organisms which were found to synthesize biotin were unaffected. On the basis of this growth-inhibition by avidin, it is predicted that *Cl. chauvei, Cl. botulinum, Cl. histolyticum, Cl. sporogenes, D. pneumoniae, L. casei* ϵ, *L. acidophilus,* and *B. brevis,* whose biotin requirements have not yet been established require biotin as a growth-essential.

[14] Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, in press.

[15] Lampen, J. O., and Peterson, W. H., *J. Am. Chem. Soc.*, 1941, **63**, 2283.

13591

In vitro Susceptibility of Pneumococci to Sulfonamide. Relationship between Size of Inoculum and Bacteriostasis.

Robert M. Pike and Elsa V. Acton. (Introduced by G. M. Mackenzie.)

From the Laboratories of the Mary Imogene Bassett Hospital, Cooperstown, N.Y.

The importance of detecting sulfapyridine-fast strains of bacteria and the need of a convenient and dependable method for measuring "fastness" are now widely recognized.[1, 2] Several methods of determining the degree to which bacteria are inhibited by sulfonamide drugs *in vitro* have been described.[2–6] While attempting to apply these methods to routine use, questions arose as to the optimum inoculum and the optimum concentration of sulfonamide to use. It has been shown that sulfanilamide will inhibit or delay the growth of hemolytic streptococci when small inocula are tested, but large inocula of susceptible strains are not inhibited even by high concentrations of the drug.[7–10] A similar quantitative relationship has been shown for pneumococci and sulfapyridine.[6, 11] Since a more complete analysis of this relationship appeared to have an important bearing on the choice of a method for testing the susceptibility of bacteria *in vitro*, we have undertaken to determine the growth-inhibiting power of a wide range of sulfapyridine concentrations for varying numbers of pneumococci.

Materials and Methods. The medium used was beef heart infusion broth, pH 7.6 to 7.8, containing 2% neopeptone (Difco) and 0.5% NaCl. Sulfapyridine in the desired concentration was dissolved in this medium which was then reautoclaved. Two percent rabbit serum was added and the broth was dispensed in 1.8 ml amounts in

1 MacLeod, C. M., and Mirick, G. S., *Am. J. Pub. Health*, 1941, **31**, 34.

2 Lowell, F. C., Strauss, E., and Finland, M., *Ann. Int. Med.*, 1940, **14**, 1001.

3 Bliss, E. A., and Long, P. H., *New England J. Med.*, 1937, **217**, 18.

4 Fleming, A., *Lancet*, 1938, **235**, 74.

5 MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

6 Spring, W. C., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1940, **19**, 163.

7 Colebrook, L., Buttle, G. A. H., and O'Meara, R. A. Q., *Lancet*, 1936, **231**, 1323.

8 Nitti, F., Bovet, D., and Depierre, F., *Compt. rend. Soc. Biol.*, 1937, **124**, 16.

9 Osgood, E. E., and Brownlee, I. E., *J. Am. Med. Assn.*, 1938, **110**, 349.

10 Long, P. H., and Bliss, E. A., *The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds*, New York, 1939, p. 97.

11 McIntosh, J., and Whitby, L. E. H., *Lancet*, 1939, **236**, 431.

13 x 100 mm tubes. Seven-hour cultures of pneumococci in rabbit blood broth were decimally diluted to 10^{-7} and 0.2 ml of each dilution added to tubes of the test medium containing sulfapyridine and to the control medium without sulfapyridine. Blood agar plates were poured with 0.5 ml of the 10^{-7} dilution in order to estimate the number of pneumococci in the inocula. Tests were incubated at a temperature of 35° to 36°C, because it was found that the growth of some strains was variable at temperatures slightly over 37°C. Readings were made at 17, 24 and 40 hours. There was seldom any visible change in serum broth after 40 hours. This reading was therefore taken as the endpoint. Although a rapid test was desired, it was found that growth at 17 and 24 hours, particularly with small inocula, was too variable to be of much value. The presence or absence of visible turbidity was recorded, and subcultures were made only to exclude the possibility of contamination.

Results. Table I records the results of growth inhibition titrations with 5 strains of *Diplococcus pneumoniae*. The largest numbers of organisms inhibited by varying concentrations of sulfapyridine, under the conditions of the test, are expressed as logarithms. At least 4 separate titrations were performed with each strain and the results were averaged. The data in Table I are shown in graphical form in Fig. 1, where the logarithms of the numbers of organisms inhibited are plotted against the concentrations of sulfapyridine, and smooth curves drawn through the points thus obtained.

TABLE I.
Logarithms of the Largest Numbers of Pneumococci Inhibited by Various Concentrations of Sulfapyridine.

Mg sulfapyridine per 100 ml broth	Strain				
	A47-III	Bog-XXIII	Bra-VII	41385-III	41382-XIV
0.1	<1.0	—	—	—	—
0.25	<1.1	—	—	—	—
0.5	2.9	<1.1	—	—	—
1	3.9	<1.3	—	—	—
2	5.1	1.9	—	—	—
3	5.1	3.1	<1.3	—	—
4	5.1	3.8	<1.8	<1.5	—
6	5.1	5.1	2.2	1.9	—
8	5.3	5.0	3.6	3.0	—
10	5.1	4.9	4.1	3.9	—
12	—	—	4.8	3.9	—
16	5.3	5.3	5.1	4.0	—
20	—	5.1	5.3	4.1	1.0
24	—	—	—	4.9	2.6
32	—	5.3	5.2	5.0	3.0
48	—	—	5.2	5.0	4.0
64	—	5.3	—	5.0	4.9

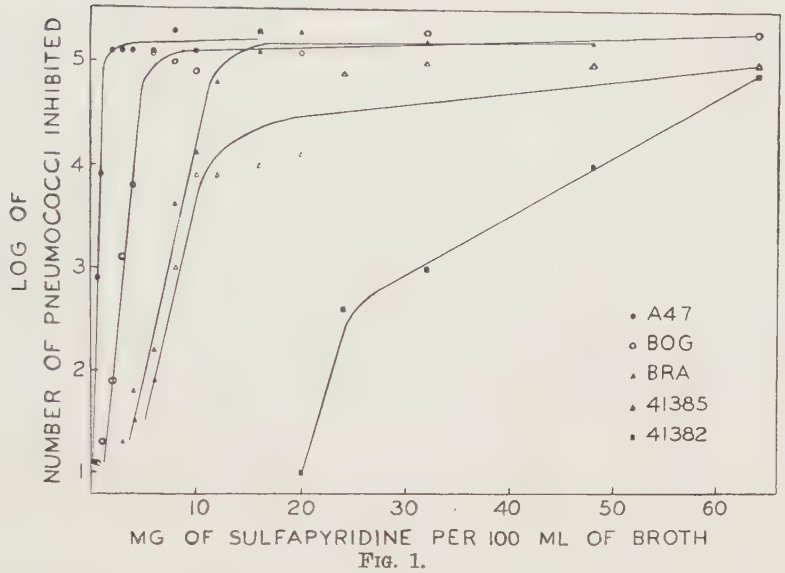


FIG. 1.

Graph showing relation of size of inoculum of five strains of pneumococci to inhibition of growth by sulfapyridine.

Differences in the susceptibility of the 5 strains are clearly illustrated.

Strains A47, Bog, and Bra were recently isolated in the course of routine bacteriological examinations. Strains 41385* and 41382* were stock strains known to be resistant to sulfapyridine. With 4 of the strains the concentration of the drug required to inhibit growth increased rapidly with the size of the inoculum up to about 100,000 organisms. The curve of this relationship appears to be an ascending straight line. Inocula above 100,000 were not inhibited regardless of the concentration of the drug and the relative susceptibility of the strain. The greater the resistance of the strain to sulfapyridine, the more gradual the slope of the curve. This was particularly noticeable with the most resistant strain, 41382. Three other resistant strains* were studied but sufficient data were not obtained to plot curves because the smallest inocula of these strains were only slightly inhibited by a concentration of 64 mg per 100 ml. Curves obtained for strains A47 and Bra with sulfathiazole were similar to those described with sulfapyridine except that less sulfathiazole than sulfapyridine was required to inhibit growth.

The results show that an attempt to titrate the sulfonamide susceptibility of a strain by testing varying inocula in a constant con-

* Furnished by the kindness of Dr. John K. Miller, New York State Department of Health, Albany.

centration of drug would have only a limited value because with any one concentration of sulfonamide an end point would be obtained for only a few of the strains which might be encountered. Using a single inoculum, such as 1000 organisms, and testing it in varying concentrations of sulfonamide, however, would theoretically give an end point with any strain. If 2 points on the logarithmic portion of the curve were determined, thereby indicating the slope of the curve and its position with reference to the abscissa, it should be possible, by interpolation, to determine the approximate concentration of drug required to inhibit the growth of any number of bacteria on this portion of the curve. Results could then be expressed in terms of the same number of bacteria for all strains tested, thereby avoiding the error due to variation in numbers of organisms present in different cultures of various strains. In our experience, the number of colonies developing from 0.2 ml of 10^{-6} dilutions of 7-hour blood broth cultures varied between 60 and 360.

In order to determine the degree of variation that might be expected in the results of repeated tests on the same strain the following experiment was performed. Twenty-five 7-hour cultures of strain Bog were decimally diluted. Two dilutions of each culture, 10^{-4} and 10^{-6}, were tested in beef heart infusion broth containing 2% rabbit serum and 1, 2, 3, 4, 5, 6, 8, and 10 mg of sulfapyridine per 100 ml. The tests were set up by 2 persons on 2 different days using different lots of sulfapyridine solutions on each day. The same lot of infusion broth was used throughout the experiment. The concentration of sulfapyridine required to inhibit visible growth for 40 hours was noted in each instance. The number of pneumococci present in the 2 inocula of each culture was estimated from colony counts. Two of the 25 tests were discarded because colonies failed to develop on poured plates. The concentration of sulfapyridine required to inhibit 1000 pneumococci was calculated algebraically from the observed data for each of the 23 remaining tests. The mean concentration of sulfapyridine required was 3.89 mg per 100 ml, with a standard deviation of 0.48. The lowest value obtained was 2.6 and the highest 4.8 mg per 100 ml. It was found that the concentration of sulfapyridine required to inhibit 1000 pneumococci could readily be estimated graphically within 0.2 mg of the figure determined algebraically.

The results of these repeated tests indicate that sulfonamide susceptibility can be determined by this method with a reasonable degree of accuracy. The expected variation is such, however, that tests should be performed at least in duplicate. The extent to which

results are reproducible depends upon the use of the same medium in comparative tests. One of our strains was tested in beef heart infusion broth with and without serum. Six mg of sulfapyridine per 100 ml were required to inhibit the growth of 24,000 pneumococci in broth containing 2% serum while the same inoculum failed to grow in a concentration of 1 mg per 100 ml without serum. Similar results were obtained with brain heart infusion broth (Difco) with and without serum. Unless serum was used, however, growth from small inocula in control tubes could not be depended upon. The increased amount of sulfapyridine required in broth containing serum was probably not due to the presence of sulfonamide inhibitor in the serum[5] but rather to the stimulation of growth by the serum.

Summary. Inhibition tests using varying numbers of pneumococci in varying concentrations of sulfapyridine and sulfathiazole indicated that except for highly resistant strains, there is a straight line relationship between the amount of drug required to inhibit growth and the number of pneumococci inoculated up to about 100,000. Larger inocula were not inhibited regardless of the amount of drug present. A method is described whereby this quantitative relationship may be applied to testing the sulfonamide susceptibility of pneumococci and expressing the results in terms of the concentration of sulfonamide required to inhibit the growth of a given number of bacteria.

13592 P

Sulfonamide Chemotherapy of Mouse Pneumonitis, Meningopneumonitis and Lymphogranuloma Venereum.

GEOFFREY RAKE, HELEN JONES AND CLARA NIGG.

From the Squibb Institute for Medical Research and the Biological Laboratories of E. R. Squibb and Sons, New Brunswick, N.J.

Within the last few years two new virus-like agents which form elementary bodies have been described. The first of these, the agent of meningo-pneumonitis, was isolated by Francis and Magill[1] from ferrets which had received nasal washings from humans with symptoms of grippe. The authors believed the agent to have been carried in their stock of ferrets. The second, the agent of mouse pneumonitis, was isolated by Nigg[2] from an apparently normal stock

1 Francis T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

2 Nigg, C., *Science*, 1942, **95**, 49.

of Swiss mice. More recent papers have shown that both these agents are related to those of the lymphogranuloma venereum-psittacosis group.[3, 4]

It has been demonstrated that the agent of lymphogranuloma venereum is one of the very few viruses or virus-like agents which is susceptible to chemotherapy with the sulfonamide drugs.[5, 6] Since two other members of this group, *viz.*, the agents of psittacosis and the very closely related one of pneumonitis,[7] are said not to be affected by the sulfonamides, it was felt that this difference in susceptibility to the action of sulfonamide drugs might be of great importance, since it might offer a clue to the mode of action of these drugs. With this in mind studies were undertaken to determine the reaction of the two new agents to sulfathiazole and sulfadiazine, the drugs found to be most effective against lymphogranuloma venereum.[6]

The results are shown in Table I. It will be seen from the experimental data with mice that both sulfathiazole and sulfadiazine were very effective against the agent of mouse pneumonitis. Both drugs, when mixed with the food (1% sulfathiazole or 0.1% sulfadiazine) were effective against at least 100 L_d 50 intranasal doses or 1 million intranasal infective doses. Moreover, the mice showed no symptoms at any time after infection and in this respect the drugs were more active than in the case of lymphogranuloma venereum where a few of the mice did show symptoms.

On the other hand, neither drug affected the action of the agent of meningo-pneumonitis whether the latter was inoculated by the intracerebral or the intranasal route. Even with larger amounts of drug (0.25% sulfadiazine, sufficient to produce an average blood level of 10 mg %, and 1.5% sulfathiazole) the intranasal infection was quite uninfluenced.

Since the results with the agent of mouse pneumonitis were obtained after intranasal infection, which is the only route by which this agent readily infects mice, and since all previous studies on chemotherapy of lymphogranuloma venereum in mice have dealt with intracerebral infection, a few experiments were made to determine the effect of both the drugs on the latter agent given

[3] Rake, G., Eaton, M. D., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 528.

[4] Eaton, M. D., Rake, G., Nigg, C., and Francis, T., to be published.

[5] Findlay, G. M., *Lancet*, 1940, **239**, 528.

[6] Jones, H., Rake, G., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 318.

[7] Eaton, M. D., Beck, M. D., and Pearson, H. E., *J. Exp. Med.*, 1941, **73**, 641.

TABLE I.
Representative Experiments Showing Action of Sulfathiazole and Sulfadiazine.

	Route of infection	Inoculum, 0.04 cc	Controls no drug	Concentration of drugs in food: Sulfadiazine 0.1%	Sulfadiazine 0.25%	Sulfathiazole 1.0%	Sulfathiazole 1.5%
Lymphogranuloma venereum	IN	10^{-1}	2,2,2,3,++	+,±,0,0,0		+,±,±,±,±	
		10^{-2}	4,5,++,+,±	0,0,0,0,0		±,0,0,0,0	
		10^{-3}	±,±,0,0,0				
		10^{-4}	+,±,0,0,0				
		10^{-5}	0,0,0,0,0				
Mouse pneumonitis	IN	10^{-1}		0,0,0,0,0		+,±,0,0,0	
		10^{-2}	7,7,7,8,+++	0,0,0,0,0		0,0,0,0,0	
		10^{-3}	7,7,++++,+				
		10^{-4}	8,++++,+++,+++				
		10^{-5}	++,++,++,+,±				
		10^{-6}	++,+,+,+,+				
		10^{-7}	+,+,+,+,±				
		10^{-8}	+,0,0,0,0				
Meningo-pneumonitis	IC	10^{-2}		4,5,5,5,5		5,5,5,6,6	
		10^{-3}	5,6,7	5,6,6,6		6,6,6,6	
		10^{-4}	7,7,7				
		10^{-5}	6,7,7,S,S				
Meningo-pneumonitis	IN	10^{-1}	2,2,3,3,3		2,2,3,3,3		2,3,3,3,3
		10^{-2}	5,6,6,9,9		3,3,3,4,4		3,3,4,4,5
		10^{-3}	7,8,8,10,++++				
		10^{-4}	10,++++,++++,++++,+++				
		10^{-5}	+++,+++,++,++,++				

IN = intranasal.
IC = intracerebral
2,3,4, etc. = days between inoculation and death.
±,+ to ++++ = degree of pulmonary involvement at autopsy 10 days after inoculation.
0 = showed no pulmonary involvement at autopsy 10 days after inoculation.
S = survived for 10 days.

by the intranasal route.[8] From the results included in the table it is apparent that the drugs are active against 100 L_d 50 or 1000 infective doses inoculated by this route.

The demonstration that at least two agents in this group, *viz.*, those of lymphogranuloma venereum and mouse pneumonitis, are susceptible to the action of sulfonamides is of further interest in that it draws attention to the possible relationship of this group to the agents of trachoma and inclusion blennorrhoea which are likewise susceptible to drugs of the same series. Further evidence bearing on this relationship will be published elsewhere.[9] The only other virus-like agent known to be susceptible to chemotherapy is that of heart-water fever,[10] which at present is classed with the rickettsiae.

It should be added that the authors do not believe that these investigations will necessarily have any bearing on chemotherapy of true virus diseases since evidence is accumulating which indicates that the members of the lymphogranuloma venereum-psittacosis group should be separated from the true viruses.

13593

Biochemical Studies of Atheromatous Animals.

GUSTAV J. MARTIN AND WILHELM C. HUEPER. (Introduced by Walter Goldfarb.)

From the Warner Institute for Therapeutic Research, New York City.

A thesis has been offered[1] suggesting that the fundamental and general causal mechanism of degenerative arterial disease is an impaired nutrition and oxygenation of the vascular wall. This thesis has been based on the production of arteriosclerotic changes by 3 general procedures: first, the use of film and emulsion-forming agents (cholesterol,[2] polyvinyl alcohol,[3] methyl cellulose,[4] etc.);

8 Shaffer, M. F., Rake, G., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 408.

9 Rake, G., Shaffer, M. F., and Thygeson, P., to be published.

10 Neitz, W. O., *J. S. African Vet. Med. Assn.*, 1940, **11**, 15.

1 Hueper, Wilhelm C., *Arch. Path.*, 1939, **28**, 510; *Medicine*, 1941, **20**, 397.

2 Anitschkow, N., Experimental Arteriosclerosis in Animals, p. 271, in Cowdry, E. V., *Arteriosclerosis*, New York, The Macmillan Co., 1933.

3 Hueper, Wilhelm C., *Arch. Path.*, 1941, **31**, 11.

4 Hueper, Wilhelm C., *Arch. Path.*, 1942, **33**, 1.

second, the use of vasculotonic (hypertonic[5] and hypotonic[6]) agents; and third, the production of abnormal hydrostatic conditions[7] in the vessels. The use of the film-forming agents offered the best possibility for a biochemical approach, since it seemed that altered oxygenation speed of erythrocytes would result if films of cholesterol or other agents of this type formed over the surfaces exposed.

In the polyvinyl alcohol experiments dogs were used exclusively. 125 cc of a 5% polyvinyl alcohol solution in normal saline were injected into the jugular vein, from which the blood was withdrawn for the various tests. Purely relative methods were devised to investigate oxygenation speeds. A blood sample of 5.0 cc was evacuated in the Van Slyke manometric apparatus at 20 mm of mercury for 5 minutes; it was then exposed to the atmosphere for 5 seconds and a manometric oxygen determination[8] was carried out immediately thereafter. All samples were treated in exactly the same manner and results on a given sample of blood could be duplicated to within 0.2 vol. %. The results are recorded in Table I. Cell sizes are included in Table I to show that the potentiality of increased exposed surface is not a factor in this determination. Cell size did

TABLE I.
Oxygenation Speed of Blood of Polyvinylized Dogs.

Dog		Vol. % O	Hb.	Cell size, u	Cell No. × 1000	Vol. % O_2/Hb
	Control	11.91	11.8	6.7	5,440	1.01
415	Immed. following 150 cc 5% PVA	12.61	10.2	6.4	4,880	1.23
	24 hr later	7.4	10.8	6.5	4,550	0.68
	Control	13.11	13.0	6.3	5,660	1.01
417	Immed. following 150 cc 5% PVA	12.87	10.9	6.1	5,040	1.18
	24 hr later	8.35	10.1	6.2	4,270	0.82
	Control	11.42	12.0	6.36	5,850	0.95
419	Immed. following 150 cc 5% PVA	10.97	12.5	6.32	6,580	0.88
	24 hr later	6.25	11.0	6.19	6,020	0.57
	Control	11.4	10.3	6.62	5,140	1.10
442	Immed. following 125 cc 5% PVA	9.8	8.8	6.62	4,330	1.11
	24 hr later	7.9	8.8	6.86	4,470	0.89
	Control	9.6	9.8	6.79	4,500	0.98
443	Immed. following 125 cc 5% PVA	7.6	7.6	6.85	3,550	1.00
	24 hr later	4.9	6.9	6.92	4,200	0.71
	Control	11.2	9.9	6.52	4,950	1.13
464	Immed. following 125 cc 5% PVA	9.6	7.7	6.45	3,920	1.25
	24 hr later	4.6	7.4	6.56	3,890	0.62

5 Hueper, Wilhelm C., and Ichniowski, C. T., *J. Lab. Clin. Med.*, 1941, **26**, 1565.

6 Hueper, Wilhelm C., and Landsberg, J. W., *Arch. Path.*, 1940, **29**, 633.

7 Moon, V. H., *Arch. Path.*, 1927, **3**, 404.

8 Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

TABLE II.
Oxygenation Speed of Erythrocytes of Cholesterolinized Rabbits.

Rabbit	Cholesterol, mg/100 cc	R.B.C. × 1000	Hb.	Vol. % O_2	O_2/Hb.
1	796	3,450	7.6	7.95	1.04
2	770	4,250	9.4	10.3	1.10
3	1020	5,050	9.4	12.0	1.26
4	1020	6,800	12.3	14.6	1.18
5	639	6,110	10.2	9.6	0.95
Control a	64	3,910	10.0	14.9	1.49
" b	42	5,200	12.5	18.0	1.44
" c	60	4,190	11.3	16.3	1.42

not vary significantly or consistently. There was a marked reduction of the O_2 uptake of the blood of the polyvinyl-treated dogs.

Identical methods were applied to the study of oxygenation speed of erythrocytes of rabbits fed cholesterol. Two grams of cholesterol in peanut oil were added to the daily ration of rabbit chow over a period of 9 to 12 weeks. The results recorded in Table II are similar to those observed with polyvinyl alcohol on dogs.

Blood chemical studies were carried out on dogs injected with polyvinyl alcohol. Three dogs received 70 cc of a 5% polyvinyl alcohol solution intraperitoneally on the first, second, sixth, eighth, tenth, thirteenth and fifteenth days of the experiment, while an untreated dog served as a control. Table III illustrates significant blood findings in a typical polyvinyl dog. All 3 of the experimental dogs exhibited an increase of N.P.N. reaching pathological values during the terminal stage. After 3 weeks there was also a uniform drop in total protein values. The markedly lowered fibrinogen values existing in 2 dogs suggest the presence of severe liver damage such as is seen in acute liver atrophy. Histological examination of the liver of these 2 animals confirmed this assumption. There were no significant deviations from the normal range in the other blood constituents determined (sodium, potassium, calcium, phosphorus, cholesterol, cholesterol esters, total lipoids, fatty acids, phospholipids, glucose) aside from the normal terminal decreases in glucose.

Plasma volume was measured in polyvinylized dogs by the method of Gregersen and Stewart[9] with the dye T-1824. 0.05 to 0.1 cc of a

TABLE III.
Protein and Non-Protein Blood Chemistry in PVA Dog.

Weeks	1	2	3	4	5	6
N.P.N., mg	31.6	29.6	27.1	41.2	100.0	120.0
Total protein, %	5.0	5.3	2.6	2.9	1.8	3.0
Fibrinogen, %	0.5	0.6	0.22	0.21	0.25	0.27

[9] Gregersen, M. I., and Stewart, J. D., *Am. J. Physiol.*, 1940, **125**, 142.

1% suspension of this dye in water was injected for each kilogram of body weight. Optical densities were read on a Hilger spectrophotometer and introduced into the formula of Gregersen and Stewart.[8] Following the injection of 50 cc of a 5% solution of polyvinyl alcohol in a series of 7 dogs, plasma volumes increased by approximately 100% during the first 24 hours. The osmotic activity of polyvinyl alcohol causes considerable amounts of tissue fluid to come into the vascular system. The macromolecular aggregates of this colloid are not rapidly and readily removed from the blood, but remain there in considerable quantities over a period of several days following a single injection. The tendency of polyvinyl alcohol to coat the intima with a film may accentuate this osmotic effect by interfering with the escape of water from the liquid component of the blood.

It would seem that cholesterol as well as polyvinyl alcohol is capable of causing an altered physico-chemical state of the blood which results in a decreased speed of oxygenation of the red cells. Christie, Phatak and Olney[10] noted a transitory lowering of oxygen saturation immediately following the injection of a 15% solution of gum acacia. They explained their observations as being due to the coating action of the gum acacia. By similar processes of elimination, it is suggested that the results herein reported may be due to a coating action of the macromolecular compounds employed. The time element was not taken into consideration by Moncke[11] who studied oxygen-combining power, not oxygenation speeds, and therefore did not obtain results comparable to those under consideration here.

Summary and Conclusions. Direct biochemical evidence is presented to show that altered or impaired oxygenation of erythrocytes occurs following the parenteral administration of polyvinyl alcohol or the oral administration of cholesterol, both film-forming agents.

The use of polyvinyl alcohol as a medium for the reëstablishment of normal osmotic pressure in the blood is considered.

10 Christie, A., Phatak, N. M., and Olney, M. B., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 670.

11 Moncke, J. V., *Am. J. Physiol.*, 1941, **132**, 529.

13594

Liver Fats and Glycogen of Hypophysectomized Rats on High Carbohydrate and High Fat Diets.*

Leo T. Samuels, Roger M. Reinecke and Howard A. Ball.

From the Division of Physiological Chemistry, Department of Physiology, University of Minnesota, Minneapolis, and the Pathological Laboratories, San Diego General Hospital, San Diego, Calif.

During balance studies on hypophysectomized rats fed high carbohydrate and high fat diets by stomach tube in amounts sufficient to maintain the body weight of control animals, it was observed that the fat-fed animals would withstand a 30-33-hour fast without showing signs of hypoglycemia. This was not true of the carbohydrate-fed hypophysectomized rats. Hypophysectomy had been performed 17 days before the fasting period was begun. The composition of the livers of rats two hours after feeding was compared with that of the livers of the fasted animals.

The livers were removed under amytal anesthesia, digested with 30% potassium hydroxide, and the glycogen precipitated with 50% ethanol. The glycogen precipitate was thoroughly washed and the combined supernatant solutions were analyzed for total fatty acids according to a modification of the method of Lemeland.[1] The non-saponifiable fraction was carefully separated in this analysis. Table I gives values for control and hypophysectomized rats on the two types of diet without fasting and after inanition.

Both Guest[2] and McKay, *et al.*,[3] found that glycogen in the livers of animals on a high carbohydrate-low protein diet disappeared rapidly. These workers found that increasing the protein content of the diet lowered the original amount of glycogen. It also lowered the rate of disappearance of this compound from the liver. In our experiments the same conserving action on glycogen seemed to be exerted by large amounts of fat in the diet, the protein representing only 15% of the calories in both diets. It can be seen that the rate

* This investigation was supported by a grant from the Committee on Research in Endocrinology, National Research Council.

Assistance in the preparation of these materials was furnished by the personnel of the Works Project Administration, Official Project No. 165-1-71-124, Sub-project No. 355.

1 Lemeland, P., *Bull. Soc. Clin. Biol.*, 1923, **5**, 110.

2 Guest, M. Mason, *J. Nutrition*, 1941, **22**, 205.

3 MacKay, Eaton M., Carne, Herbert O., Wick, Arne N., and Visscher, Frank E., *J. Biol. Chem.*, 1941, **141**, 889.

TABLE I.
Liver Lipids and Glycogen in Hypophysectomized and Control Rats Fed Equicaloric Amounts of High Carbohydrate and High Fat Diets.

	High fat diet		High carbohydrate diet	
	Hypophysectomized	Controls	Hypophysectomized	Controls
Not Fasted:				
No. rats	6	9	6	9
Fatty acids mg/100 g body wt	245	585	98	241
Non-saponifiable mg/100 g body wt	16.8	10.9	10.0	8.3
Glycogen mg/100 g body wt	51.3	95.6	108	140
Fasted 30-33 hours:				
No. rats	14	15	9	9
Fatty acids mg/100 g body wt	127	529	87	224
Non-saponifiable mg/100 g body wt	19.3	12.0	9.3	7.7
Glycogen	9.1	19.7	0.16	2.5
Glycogen lost during fasting mg/100 g body wt	42.2	75.9	107.8	137.5
Mean mol. wt. fatty acids	290 ± 1.8	286 ± 1.9	294 ± 2.0	279 ± 1.9
% fat in carcass	10.80	9.53	13.23	8.14

of disappearance of glycogen in the fat-fed animals was much less than in the carbohydrate-fed groups. The difference in rate of disappearance was almost the same whether the animals were hypophysectomized or not (65.6 mg and 61.6 mg in 30 hours, respectively). The glycogen-sparing action of a fat diet did not appear to be influenced by pituitary removal.

The failure of the fat-fed hypophysectomized rats to show signs of hypoglycemia was probably due to the decreased utilization of carbohydrate during fasting and the greater amount of glycogen consequently remaining in the liver.

On both diets the hypophysectomized rats showed in the liver only approximately half the amount of fat which was present in this organ in the controls. On the other hand, the amount of fat in the carcass, other than the liver, was slightly higher. This would seem to agree with the view that the mobilization of peripheral fat is increased by pituitary secretion.

The mean molecular weights of the liver fats found in the control animals indicate that the average length of carbon chains was less on the carbohydrate diet. This agrees with Longenecker's observations[4] on storage fat obtained from carbohydrate. In the hypo-

[4] Longenecker, Herbert E., *J. Biol. Chem.*, 1939, **128**, 645.

physectomized rats there was no significant difference in the average molecular weight between the 2 groups, although the animals on the carbohydrate diet had liver fat with the highest molecular weight of the 4 groups. These animals also had the least amount of fat in the liver. The difference may be due to this being largely a residuum of phospholipid.

Summary. On a high fat diet the glycogen content of the liver was originally lower than on a high carbohydrate diet, but it did not disappear so rapidly from the liver. Hypophysectomized rats had less liver fat and more body fat than controls on the same caloric intake of a given diet. They showed the same conserving action of the fat diet on glycogen. The molecular weight of the liver fatty acids of the control rats on a high carbohydrate diet was significantly lower than those of the 3 other groups.

13595 P

Response of Bone Marrow in AKH and RFI Mice to Nucleic Acid.*

JACKSON NEAVLES. (Introduced by Robert A. Moore.)

From the Department of Pathology, Washington University School of Medicine, St. Louis, Mo.

Furth and his associates[1] and MacDowell and Richter[2] and others have shown that a leukemia similar to the human disease occurs spontaneously in mice, and that by inbreeding of selected individuals the percentage incidence of the condition may be increased to as high as 80%. Furth has developed two strains known as the Akh and the Rfi. The Akh strain carries lymphoid leukemia, and 60% to 80% of all animals which live to be 8 months of age suffer from the disease. The Rfi strain carries myeloid leukemia, and in somewhat older mice from 1% to 2% develop this variety of leukemia. This preliminary report is concerned with attempts to demonstrate additional constitutional differences between these two strains of mice. The most logical approach appeared to be some test of the response of the bone marrow to stimulation. Menkin[3] showed that

* This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

1 *Am. J. Cancer,* 1937, **31**, 276.

2 *Cancer Probl. Symposium,* September, 1937, 42-44.

3 Menkin, V., *Am. J. Path.,* 1940, **16**, 13.

sterile inflammatory exudates produced in the pleural cavity by the injection of turpentine contained a substance that provoked leukocytosis when injected into other animals.

Pleural exudates were collected according to the directions of Menkin, and the globulins were precipitated by half-saturated ammonium sulfate. Injections of from 20 to 50 mg of this material caused a leukocytosis of from 18 to 40 thousand in Akh mice and a leukopenia of 6 to 10 thousand in Rfi mice, if the blood counts were made 4 hours after the injection. The average normal white blood count was 11,500 in the Rfi, 12,400 in the Akh. Further chemical study showed that the active principle was precipitated by dilute acids and by ethyl alcohol. It, therefore, seemed possible that the activity was due to some substance associated with the nucleoproteins. Accordingly, 10 to 15 mg of thymonucleic acid were injected intraperitoneally into the 2 strains of mice. The results were essentially the same as those obtained with the exudate. The average white blood cell count after 4 hours in Akh mice was 32,150 and in Rfi mice 9,900. A similar but less striking response was secured with yeast nucleic acid, that is, a leukocytosis in the Akh animals and no change or slight leukocytosis in the Rfi animals.

The above results are based entirely on determinations made 4 hours after the injection of the stimulatory substance. More complete investigation over a 20-hour period showed that the Rfi animals which at the end of 4, 6, and 8 hours had a leukopenia, developed a leukocytosis at the fourteenth and eighteenth hour. Thus the 2 animals both responded with a leukocytosis, immediate in the case of the Akh animal and delayed in the Rfi animal. As a point of further investigation it seemed possible that this difference was due to a difference in the ability of the two strains to hydrolyze the nucleic acid and liberate the actual substance producing the leukocytosis. Thymonucleic acid was digested with duodenal mucosa at a pH of 8.5 for 24 hours. This digest gave an immediate leukocytosis in both strains. Similarly, the two pure chemical substances, adenine and guanine, in 25 mg amounts, produced a leukocytosis in both strains. It should, however, be pointed out that 4 of the 20 animals injected with adenine and guanine gave no leukocytosis.

With this information regarding the effect of the split products of nucleic acid, it became desirable to study the relative amount of the nuclease enzymes in the blood and tissues. These experiments were carried out by determination of the amount of inorganic phosphate liberated from 100 mg of thymonucleic acid at a pH of 7.4 during the course of incubation for 2½ hours. There was no dif-

ference in the amount of enzyme in the liver, but preliminary experiments with blood and with the spleen indicated that almost twice as much inorganic phosphate was liberated in experiments with Akh animals as with Rfi animals.

Finally it is worthy of note that the leukocytosis produced in the Akh strain is primarily a segmented-cell type of response. A typical hemogram four hours after the injection of thymonucleic acid was: lymphocytes, 13; monocytes, 1; segs, 63; stabs, 14; juveniles, 5; eosinophiles, 2; and basket cells, 2.

It should be emphasized that the experiments deal with the response of the bone marrow, while the type of leukemia in the Akh animal is lymphoid. We have not been able as yet to establish any connection between the type of response and the subsequent development of leukemia, in a given animal, but experiments along this line are under way.

Summary. By comparison of the response of the bone marrow to the injection of nucleic acid and its split products a difference has been demonstrated between two strains of mice, Akh and Rfi, the former of which carries lymphoid leukemia and the latter myeloid leukemia.

13596

Promin in Treatment of Experimental Tuberculosis.

M. MAXIM STEINBACH AND CHARLES J. DUCA.

From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York City.

Promin* is the sodium salt of p,p[1]-diaminodiphenylsoulfone-N,N[1]—(dextrose sulfonate).

Greey, MacLaren, and Lucas[1] reported that promin is effective in streptococcus infections in mice. Feldman, Hinshaw, and Moses[2] reported that it exerts an impressively beneficial effect on the course of experimental tuberculosis in guinea pigs, even when[3, 4] treatment

* Supplied through the courtesy of Parke, Davis and Co., Detroit.

1 Greey, P. H., MacLaren, D. B., and Lucas, C. C., *Can. Med. Assn. J.*, 1939, **40**, 319.

2 Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Proc. Staff Meet. Mayo Clin.*, 1940, **15**, 695.

3 Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Ibid.*, 1941, **16**, 187.

4 Hinshaw, H. C., and Feldman, W. H., *J. A. M. A.*, 1941, **117**, 1066.

was delayed until 6 weeks after infection. These authors administered the drug orally. Woodruff[5] reported that promin was not beneficial when administered subcutaneously to tuberculous guinea pigs. Toomey and Roach[6] obtained favorable results in acute streptococcus infections and have summarized the toxic reactions observed in 150 patients who were given large doses daily by the intravenous route. Coggeshall, Maier, and Best[7] have described the beneficial effects of promin on certain types of naturally acquired malaria in man, using large doses given intravenously for a short period.

We think it is noteworthy that every report on the promin treatment of humans indicates that the toxic reactions are serious only when the drug is administered orally. We also have had this experience while treating patients at the Montefiore Hospital. There are theoretical grounds for believing that promin is hydrolyzed in the stomach, splitting off the glucose groups and releasing the toxic parent diaminodiphenylsulfone. This substance has given favorable results in avian tuberculosis of rabbits,[8, 9] and we are beginning a study of its effect on mammalian tuberculosis in guinea pigs. Because of these considerations, we decided to use the subcutaneous route in our experiments.

Experimental. 1. Four experiments with 2 strains of human tubercle bacilli (H37 and a recently isolated strain) showed that when promin is added to Sauton's medium in concentration of 40 mg %, it inhibits the growth of tubercle bacilli for about 30 days, after which time the organisms began to grow. This phenomenon is being investigated. There was no change in morphology, staining reactions, or perhaps virulence, in organisms that had remained under these conditions for 30 days. Guinea pig inoculation with these cultures produced invasive tuberculosis.

2. Nineteen tuberculin-negative guinea pigs, average weight 400 g, were infected subcutaneously in the groin with a suspension of organisms extracted from fresh human sputum by the NaOH concentration method, and containing from 3 to 5 acid-fast bacilli per oil immersion field. Eight of these animals served as controls. The other 11 were each given three daily doses of 140 mg promin in 40% aqueous solution subcutaneously, starting on the day of infection. When an animal in either group died, an animal from the other group was sacrificed for purposes of comparison.

5 In Proc. Am. Soc. Bact. and Path., *Am. J. Path.*, 1941, **17**, 578.

6 Toomey, J. A., and Roach, F. E., *J. Ped.*, 1941, **18**, 1.

7 Coggeshall, L. T., Maier, J., and Best, C. A., *J. A. M. A.*, 1941, **117**, 1077.

8 Rist, N., *C. R. Soc. Biol.*, 1939, **130**, 976.

9 Rist, N., Bloch, F., and Hamon, V., *Ann. Inst. Past.*, 1940, **64**, 203.

Results. For the first 3 weeks, the deaths occurring were due to intercurrent infection, and there was little difference noted in the amount of tuberculosis in the 2 groups. After this time, however, there was marked spread of the disease in the control animals, while this spread was absent or minimal in the treated ones. When the experiment was terminated 7 weeks after infection, 5 animals in each group survived. Upon autopsy the controls showed maximal tuberculosis of all organs, while the treated animals showed little or no progress from the picture seen at the 3-week stage.

These results encouraged us to repeat the experiment with a larger series of animals.

3. Thirty-seven male tuberculin-negative guinea pigs, average weight 500 g, were infected subcutaneously in the groin with .001 mg of a culture of the organism used in Experiment 2. Starting on the day of infection, 20 of these were each given 160 mg of promin in 40% aqueous solution subcutaneously 3 times daily. The remaining 17 animals served as controls. With 2 exceptions, no animals were killed until the end of the experiment. The entire group of animals was tuberculin tested 2 weeks after infection. The blood levels of promin were estimated in the treated animals at various times in the day. All animals were weighed once a week, and upon autopsy, the amount of disease was noted on a scale from 0 to 4, the lungs, liver, and spleen were weighed, and representative portions of the lungs, liver, spleen, and lymph gland were obtained for histological studies.

Results. Tuberculin Tests. All 37 animals reacted positively to 0.1 mg O.T. intracutaneously.

Promin Level of Blood. This ranged between 5 and 44 mg %, the average being 18 mg %. The lowest values were found between the evening (6 p.m.) and the morning (8:30 a.m.) doses; the highest value was found an hour after any dose.

Weight. The treated animals lost about 25% more than the controls. We believe a large part of this difference may be due to the greater amount of handling to which the treated animals were subjected, as well as to the toxic effects of the drug, which will be briefly described below.

Survival Time and Autopsy Findings. Our observations on these topics have been pictured in Chart 1. Reference to this chart shows that one control and 3 treated animals died of intercurrent disease shortly after infection. The remaining 16 controls show the typical survival times of a group of animals infected with a standard dose of tubercle bacilli and kept under identical conditions. Two animals

died of tuberculosis relatively early, 12 died between 80 and 165 days after infection, and 2 lived for longer periods, namely 185 and 250 days after infection, when they died of tuberculosis. Only 3 of these 16 controls showed less than maximal tuberculous involvement. When we examine the findings in the treated animals, we see that this normal pattern is greatly disarranged. Deaths are only one-third as frequent as in the controls. Seven of the treated animals were living and apparently in good health on the 271st day after infection, when they were sacrificed to terminate the experiment. Only 2 of the treated animals showed maximal tuberculous involvement. Six of them showed minimal tuberculosis and died from some other cause, while 9 showed moderate or severe involvement. The averages of the autopsy scores of the 2 groups were 3.5 for the controls, and 2.0 for the promin-treated animals. Furthermore, after the hundredth day after infection, every treated guinea pig showed gross evidence of fibrosis. This occurred in about half of the controls, but was not as marked or as consistent.

Histologic. Examination of sections of the organs confirmed the gross autopsy findings; the treated animals had less tuberculosis and it was more fibrotic and chronic in type than that seen in the controls. Sections stained for acid-fast organisms showed the tissues of the treated animals to contain about ⅔ of the number of acid-fast bacilli found in the controls. However, all of the treated animals had tuberculosis.

Toxic Reactions. The injections were apparently quite painful to the animals, who cried and scratched the site of injection for 2 or 3 minutes. The injection caused ulcers in about 50% of the animals.

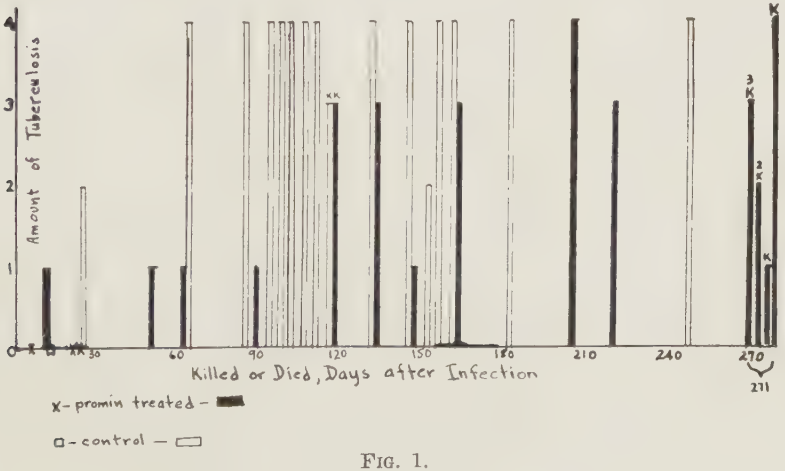


FIG. 1.

A few of these penetrated the muscle and attained a diameter of 1.5 cm. However, all of them healed in 2 or 3 weeks without secondary infection. Seven of the treated animals showed varying grades of fatty degeneration of the liver, while this condition was found in only one control guinea pig. However, because of the small numbers involved, we do not wish to draw conclusions from this latter observation.

Discussion. From the evidence presented above, we think it reasonable to conclude that promin exerts a retarding effect on the course of experimental tuberculosis in guinea pigs. Under the conditions of our experiments, this beneficial effect is manifested by a retardation of the spread and multiplication of tubercle bacilli in the animals. While these results are far superior to those which we have obtained with any other therapeutic agent in experimental tuberculosis, they fall far short of the wonderful effects reported by Feldman and his coworkers. These differences may be explained in part by variations in experimental procedure in such external and internal factors as route of administration of the drug, organism used, and breed of guinea pig. However, an adequate chemotherapeutic agent for humans should be able to cope with bacteria of wide differences in virulence, and should be of help in individuals of various constitutional makeups and from a wide variety of environments. Nevertheless the results reported, in our opinion, indicate that promin should have an exhaustive and prolonged study in the treatment of human tuberculosis.

Summary and Conclusions. 1. Promin exerts a bacteriostatic effect on tubercle bacilli *in vitro*. 2. Promin exerts a retarding effect on guinea pig tuberculosis, but does not cure the disease. 3. The effects of promin on human tuberculosis are being investigated.

13597 P

Complementing Action of Eserine and Acid in Neurohumoral Activation.

ROBERT GESELL, CHARLES R. BRASSFIELD AND ELWOOD T. HANSEN.
From the Department of Physiology, University of Michigan, Ann Arbor, Mich.

Since eserine permits a greater accumulation of acetylcholine at junctional points of activated ganglion cells, muscle fibers and other

end organs, and granting the same for increased acidity, it is to be anticipated that eserine and carbon dioxide would complement each other in their effects upon the body.[1-6] This preconceived idea was put to a simple test in anesthetized dogs connected with rebreathing tanks for alternate administration of room air and carbon dioxide mixtures.

The cardio-inhibitory reflex initiated by faradic stimulation of the superior laryngeal nerve served as the physiological indicator. The superior laryngeal nerve was stimulated for a period of 5 seconds at intervals of 2 minutes with faradic shocks of uniform intensity.

It was demonstrated, by the administration of a 10% mixture of carbon dioxide in 40% oxygen, that hypercapnia increased the reflexogenic inhibition of the heart. Intravenous injection of eserine superimposed upon a continuing hypercapnia increased and prolonged the reflexogenic cardio-inhibition still more. Return to room air while the effects of eserine were still evident produced an abrupt diminution in the degree of cardio-inhibition. These results permit 4 conclusions. 1. Hypercapnia potentiates the cardio-inhibitory reflex initiated by stimulation of the superior laryngeal nerve. 2. Eserine produces a comparable potentiation. 3. The potentiation produced by eserine is addible to that of hypercapnia. 4. The potentiating action of hypercapnia is subtractible from that of eserine.

Because the frequency of the heart beat is probably the end effect of the influences of many junctional stations, parasympathetic, sympathetic and central, a quantitative allocation of the acid-neurohumoral effects at the several stations is at present impossible. Nor are the effects of acid to be limited to its preservative action on physiologically deposited acetylcholine alone. There are at least two other possibilities, which we should like to suggest in a tentative way. One is the increased elimination of potassium during asphyxia.[7,8] The intimate interrelation of the potassium ion and acetylcholine has been described.[9,10] The other is the depressant action of cH upon the

1 Gesell, R., Brassfield, C. R., and Hamilton, M. A., *Univ. Hosp. Bull.* (Mich.), 1941, **7**, 94.

2 Gesell, R., Brassfield, C. R., and Hansen, E. T., *Univ. Hosp. Bull.* (Mich.), 1941, **7**, 105.

3 Hansen, E. T., Worzniak, J. J., and Gesell, R., *Proc., Am. J. Physiol.*, in press.

4 Brassfield, C. R., and Gesell, R., *Proc., Am. J. Physiol.*, in press.

5 Mason, A., and Gesell, R., *Proc., Am. J. Physiol.*, in press.

6 Gesell, R., Brassfield, C. R., and Hansen, E. T., *Proc., Am. J. Physiol.*, in press.

7 Cattell, M., and Ewin, H., *J. Biol. Chem.*, 1938, **126**, 633.

8 Fenn, W. O., *Physiol. Rev.*, 1940, **20**, 377.

9 Brown, G. L., and Feldberg, W., *J. Physiol.*, 1936, **86**, 290.

10 Brown, G. L., and Feldberg, W., *J. Physiol.*, 1936, **86**, 10P.

physiological action of adrenaline.[11] The latter offers most interesting possibilities in the dual sympathetic and parasympathetic controls of the body of which cardiac regulation is an example. It strongly suggests a reciprocating chemical control built upon physiological variation of the hydrogen ion concentration. Specifically, a rising cH increasing the parasympathetic inhibitory action might be supported by a diminishing intensity of sympathetic excitatory action, and a fall in cH would be associated with a converse reciprocal interaction. Central neuroreciprocal action between the excitatory and inhibitory cardiac centers would be complemented by an outlying chemical reciprocation.

On the assumption that carbon dioxide can substitute for eserine and related compounds for producing experimental effects in those systems of the body where acetylcholine is normally deposited, it is deemed advisable to reinvestigate many of the fundamental researches in neurohumoral physiology. In the light of normal and pathological fluctuations in acid-base equilibrium and the practical applications which may arise, this course is imperative. The valuable studies of Cannon and Rosenblueth[12] and their associates on the five stages of stimulation where physostigmine has such profound effects is but a single example. Will carbon dioxide produce comparable effects to those of physostigmine on these phases of stimulation? Is an increasing muscle cH a possible explanation of the fifth stage? These are merely illustrations of the many questions that may be raised to test the application of the acid neurohumoral mechanism of activation in the body.

13598

Influence of Intensity of White Light upon Pupil Diameter of the Human and of the Rabbit.

I. H. WAGMAN AND L. MATTHEW NATHANSON. (Introduced by J. M. D. Olmsted.)

From the Division of Physiology, University of California Medical School, Berkeley.

Hecht and Pirenne[1] attempted to measure the minimum visual threshold of the nocturnal long-eared owl by comparing the inten-

[11] Andrus, E. C., *J. Physiol.*, 1924, **59**, 361.

[12] Cannon, W. B., and Rosenblueth, A., *Am. J. Physiol.*, 1937, **119**, 221.

[1] Hecht, S., and Pirenne, M. H., *J. Gen. Physiol.*, 1940, **23**, 709.

sity of monochromatic light necessary to cause a pupillary constriction of 0.5 mm in the dark adapted owl with the intensity necessary to do the same in the human, thus assuming that the relationship between thresholds for intensity discrimination and pupillary constriction is the same in both species. Their data for the human pupil were taken from measurements on 6 subjects made by Reeves in 1918 and 1920.[2]

Reeves' data, although unique, cannot be taken as too accurate at low intensities because of too few measurements. Reeves took as the first point on his curve the size of the pupil after 15 minutes in the dark. He assumed this to be its size at the visual threshold which was assigned an arbitrary value of –6 log intensity units. At his next point, –3.8 log intensity units, the pupil already shows a distinct contraction. One cannot be sure at what point in the curve the pupil starts to contract, and consequently any reasonably accurate judgment cannot be made as to the relationship between visual threshold and threshold for pupillary contraction.

Reeves' work is here repeated more carefully, using as subjects both the human and the rabbit, and comparison is made between their pupillary thresholds. If Hecht and Pirenne are justified in their assumptions, then the data here obtained may offer information regarding the visual threshold in the rabbit gained by an objective method.

The method used is to be described in detail elsewhere. It is a modification of the infrared photographic method described by Gullberg, Olmsted, and Wagman,[3] and enables the pupil diameter to be measured under any condition of light or dark adaptation, as well as the subjective visual threshold. The subject was dark adapted for a period of time ranging between 20 and 30 minutes. With the human (6 subjects) at the end of this time, the subjective visual threshold was measured and a series of photographs taken with this light in the eye. With the rabbit (10 subjects) the first photographs were taken at a brightness of 6.14×10^{-5} foot-lamberts, a brightness which was found not to affect the dark adapted pupil. The intensity of light was then increased in definite steps and a series of photographs was taken at each intensity at intervals of 10, 20, 30, 45 and 60 seconds. Sixty seconds are enough to insure complete adaptation at each intensity. Periods of rest in the dark between changes of intensity were found to be unnecessary, since

2 Reeves, P., *Psychol. Rev.*, 1918, **25**, 330; *J. Opt. Soc. Am.*, 1920, **4**, 35.

3 Gullberg, J. E., Olmsted, J. M. D., and Wagman, I. H., *Am. J. Physiol.*, 1938, **122**, 160.

the state of light adaptation readily changed its level at each intensity (*cf* Hecht and Schlaer[4]).

The field of light, which was 16.6° visual angle, was centrally fixated for the human. For the rabbit, the spot was adjusted so that it appeared to be central in position. The rabbit was rigidly held in place after adjustment by means of a specially constructed holder. No anesthetic was necessary with this device, which held the rabbit securely and quietly in position in a comfortable manner with no previous struggling. The head of the human subject was held in place by a chin rest and upright bar and eyepiece.

The pupil diameter was measured at nine intensities over a range of about 9 log units (from 2.5×10^{-7} foot-lamberts to 105.5 foot-lamberts) for the human, and eight intensities over a range of about 7 log units (from 6.14×10^{-5} to 206.6 foot-lamberts) for the rabbit.

If the light is kept in the eye, the pupil dilates slightly following its initial rapid constriction. This dilatation is followed by a secondary constriction. There is always a continual small fluctuation in the pupil, no matter what the state of adaptation (Reeves[2] and Laurens[5]). The changes, however, are small enough to be ignored.

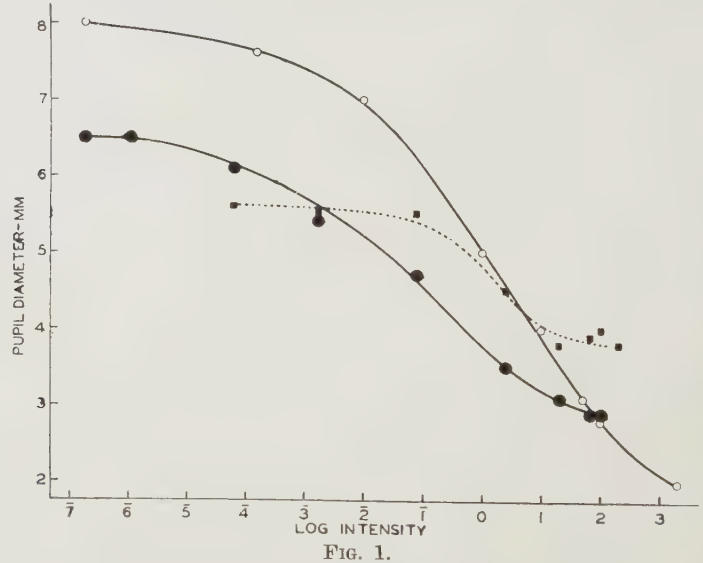


FIG. 1.

Curves showing the relationship between the intensity of white light and the pupil diameter for both the human subject and the rabbit.

● The present measurements on humans.

○ Reeves' measurements (1918, 1920) on humans.

■ The present measurements on rabbits.

[4] Hecht, S., and Schlaer, S., *J. Gen. Physiol.*, 1936, **19**, 965.

[5] Laurens, H., *Am. J. Physiol.*, 1923, **64**, 97.

The times in which the pupil closed to the minimum with various intensities were always the same.

It was found here that the pupil in both humans and rabbits reached its minimum in about 5 seconds, and thereafter fluctuated closely around that value (*cf.* Laurens and Reeves). It cannot be said whether or not pupillary size after a given exposure time longer than 5 seconds is more representative of the state of adaptation than its size after any other length of exposure. Accordingly, the pupillary sizes determined after 10, 20, 30, 45 and 60 seconds' exposure to a certain intensity were average to give the value representative of that intensity. The same curve is always obtained when the measurements are taken for any one of these intervals.

The first point in our data for the human subjects (the filled in circles of Fig. 1) is taken at the average of all the threshold values (–6.74 log I) determined subjectively by means of the adapting instrument. The range of these threshold values for the 7 subjects was from –6.28 to –7.13 or 0.85 log units.

The average pupil diameter with the threshold light in the eye is the same as that found after 20 minutes in darkness. From the curve it is evident that the pupil does not start to contract until the brightness is raised by one log unit or until it is 10 times greater than the threshold brightness.

We have arbitrarily placed the first point of Reeves' data (open circles, Fig. 1) at the same place as our own average threshold value, instead of at –6.0 log units which he arbitrarily assigned. No practical difficulties are encountered in comparing his curves with ours although his measurements were in milli-lamberts instead of foot-lamberts. Since the curves in their upper halves run parallel courses, the pupils measured by Reeves would probably show the start of contraction at the same point as ours do. If we take a constant amount of pupillary contraction (*i. e.,* 0.5 mm as Hecht and Pirenne did) to make comparisons, the similarity between the two curves is more striking. Thus our data show that a 0.5 mm contraction occurs at –3.75 log units, which is 2.99 log units more, or 1000 times more than the average threshold value. Reeves' curve shows that a 0.5 mm contraction occurs at about –3.35 log units, which is 3.39 log units more than our average threshold values. Since the latter is merely a guess when applied to Reeves' data, the agreement is surprisingly good. The difference between 3.39 and 2.99 or 0.40 log units is well within the normal variation of subjective thresholds as measured by us and by Hecht and Mandelbaum.[6]

[6] Hecht, S., and Mandelbaum, J., *J. A. M. A.*, 1939, **112**, 1910.

This similarity between the two sets of data holds for any pupillary constriction up to about 3 mm. We think that the discrepancy from then on is not real, since the brightness measurements made by Reeves do not seem to be accurate at the higher intensities. Although he is not specific, he does say the pupils were measured at brightnesses obtained from direct sunlight or reflections from a white surface. Furthermore, his last point at 2000 milli-lamberts (or 3.3 log units) was obtained from one subject, the one having the second smallest pupil. Since light of this intensity is painful, measurements are extremely difficult to make in such a bright light.

Our results agree with those of Reeves in showing that the pupillary constriction for the human is very slight above an intensity of approximately 100 foot-lamberts.

The average curve for the 10 rabbits is the dotted curve in Fig. 1. The pupil does not start to constrict until an intensity of about −1.5 log units is reached, which is about 4 log units or 10,000 times greater than the intensity necessary to cause a just perceptible contraction of the human pupil. A contraction of 0.5 mm in the rabbit occurs at −0.40 log units or at an intensity of 3.35 log units or about 2200 times greater than the intensity necessary to do the same in the human. We prefer to use, as did Hecht and Pirenne, this latter method of comparison since the determination of the point where the pupil just starts to contract is much more inexact. In any event, the error is less than 1.0 log unit, within the order of variation found in subjective measurements on humans.

If Hecht and Pirenne's assumption is correct that the relationship between absolute threshold and a constant amount of pupillary constriction is constant for different species of animals, then our results show that the rabbit's absolute threshold is 2,200 times greater than man's and 22,000 times greater than the owl's.

13599 P

Acute Toxicity for Mice of Phthalic Acid and Certain Derivatives.*

Harold C. Hodge, Max R. Goldstein and Margaret Wrightington.

From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, N.Y.

Although a number of reports have appeared describing the toxic properties of diethyl phthalate,[1-5] a single report is available on dimethyl phthalate[6] and none on phthalic acid, sodium phthalate, dibutyl phthalate or dioctanol 2 phthalate.

Acute Toxicity for Mice. Intraperitoneal injections of the various compounds were given to 3-months-old, male, white mice. Six mice were kept in one cage; 4 such groups were simultaneously injected with a given dosage using a tuberculin syringe. The mice ranged from 18-22 g in weight; average weight 20 g. The mice were observed periodically and the total number dead at the end of 24 hours was taken as the kill for a given dosage. All of the mice surviving 24 hours recovered (with the few exceptions noted below).

Phthalic Acid. The phthalic acid was given as a saturated solution which was shown to contain 0.01 g of phthalic acid per ml. Higher doses than 12 mg per mouse were not feasible since this dosage required the intraperitoneal injection of 1.2 ml which is a large volume compared to the size of the peritoneal cavity. Only with phthalic acid were any observed to die later than 24 hours after administration. The deaths in the second day were as follows: with a dose of 6 mg, late deaths, none; with 8 mg, 2; with 10 mg, none; with 12 mg, 2. The dose required to kill the average mouse (L.D. 50) was calculated† to be 0.55 g per kg body weight (Table 1A). There was some cyanosis noted; the mice hopped about spasmodically, standing

* This work was supported in part by a grant from the Carnegie Corporation of New York.

The assistance of Raymond Kesel, Charles Levine and Dr. Leon A. Heppel is gratefully acknowledged.

1 St. George, *Am. J. Clin. Path.*, 1931, **7**, 69.

2 McNally, *Ind. Med.*, 1938, **7**, 295.

3 Blickensdorfer and Templeton, *Am. Pharm. Assn.*, 1930, **19**, 1179.

4 Flury and Wirth, *Arch. Gewerbepath. Gewerbehyg.*, 1933, **5**, 1.

5 Martin and Salmon, *J. Agric. Sci.*, 1934, **24**, 469.

6 Donley, *J. Ind. Hyg. Toxic.*, 1937, **7**, 69.

† The statistical procedure was taken from Bliss, *Ann. Appl. Biol.*, 1935, **22**, 134.

on their hind legs. In certain mice, autopsy showed an apparent subcutaneous hemorrhage in the nasal region; the livers exhibited white spots of unknown etiology.

Sodium Phthalate. The average fatal dose was calculated as 2.1 g per kg body weight (Table 1B); this represents a reduction of toxicity as compared to phthalic acid of about 75%. Cyanosis, convulsive movements and hopping were observed in the treated mice. On autopsy, many mice exhibited congested lungs, hemorrhages into the nasal and oral cavities and pale livers.

Dimethyl Phthalate. The dose required to kill the average mouse was calculated to be 2.4 ml per kg body weight (Table 1C). It was noted that after injection of dimethyl phthalate the mice uniformly developed a marked cyanosis. Their breathing became very rapid

TABLE I.
Data on Dosage and Percentage Mortality.

No. mice	Dosage	No. dead	Mortality %
	A—*Phthalic acid:* 113 mice.		
24	6 mg	1	4
24	8	11	44
41	10	19	46
24	12	12	50
For probit kill—5.00, L.D. 50—11 mg.			
	B—*Sodium phthalate:* 168 mice.		
24	27 mg	4	17
24	33	5	21
24	40	13	54
24	47	13	54
24	53	20	83
24	60	16	67
24	67	20	83
For probit kill—5.00, L.D. 50—42 mg.			
	C—*Dimethyl phthalate:* 120 mice.		
24	.03 ml	0	0
24	.04	7	29
24	.05	13	54
24	.06	19	79
24	.07	24	100
For probit kill—5.00, L.D. 50—0.047 cc.			
	D—*Di n-butyl phthalate:* 97 mice.		
30	.05 ml	1	3
30	.10	13	43
25	.15	25	100
6	.20	6	100
6	.40	6	100
For probit kill—5.00, L.D. 50—0.11 cc.			
	E—*Dioctanol 2 phthalate:* 72 mice.		
24	.5 ml	4	17
24	.8	7	29
24	1.0	15	62
For probit kill—5.00, L.D. 50—0.92 cc.			

and they developed so severe a weakness that they were unable to walk. They became stuporous and remained quiet for some hours.

Di-n-butyl Phthalate. The L.D. 50 was found to be 5.5 ml per kg body weight (Table 1D).

Dioctanol 2 Phthalate. It was found by statistical analysis that the lethal dose for the average mouse is 46 ml of dioctanol 2 phthalate per kg body weight (Table 1E). The mice lived for several hours before any died.

Summary. The L.D. 50 doses for the mice used are as follows: Phthalic acid, 0.011 g; sodium phthalate, 0.042 g; dimethyl phthalate, 0.05 ml; dibutyl phthalate, 0.11 ml; and dioctanol 2 phthalate, 0.92 ml. Compared to phthalic acid, the L.D. 50s of the compounds are as follows: Phthalic acid, 1, sodium phthalate, 4; dimethyl phthalate, 5; dibutyl phthalate, 10; dioctanol 2 phthalate, 84.

13600

Bomskov Reports on Thymus Mediation of Pituitary Function.

B. B. WELLS, OSCAR RIDDLE AND H. N. MARVIN.

From the Carnegie Institution, Department of Genetics, Cold Spring Harbor, N.Y.

A series of papers by Bomskov and coworkers[1-5] in Freiburg reports the preparation and extensive investigation of an ether-soluble extract of the thymus. This extract was said to be "diabetogenic" (in rats, guinea pigs, pigeons) in that it showed marked glycogenolytic and hyperglycemic actions. These investigators claimed that a diabetogenic fraction of the anterior pituitary gland failed to lower the liver glycogen in rats whose thymus tissue had been destroyed by Roentgen irradiation.[1] They, therefore, postulated, and later claim to have shown, that the diabetogenic pituitary fraction is thymotrophic and exerts its recognized physiologic actions through stimulating the production of a hormone in the thymus. As the result of other work Bomskov and coworkers[2, 3] indicate that their diabetogenic-thymotrophic fraction from the pituitary gland is identical with growth hormone, and that physiologic effects of the latter

1 Bomskov, C., and Sladovic, L., *Deutsch. Med. Wochschr.*, 1940, **66**, 589.
2 Bomskov, C., and Hölscher, B., *Z. Klin. Med.*, 1940, **137**, 745.
3 Bomskov, C., and Sladovic, L., *Pfluger's Arch.*, 1940, **243**, 611.
4 Bomskov, C., and Brachat, F., *Endokrinologie*, 1940, **23**, 145.
5 Bomskov, C., and Karl-Heinz, K., *Pfluger's Arch.*, 1940, **243**, 623.

pituitary substance(s) are thus also mediated by the thymus hormone. In still other studies[4, 5] it is reported that a lymphocytosis and leucocytosis regularly follow the administration of thymus hormone to rats. On the basis of this finding, and of certain histological observations on thymus tissue, they develop the theory that thymus hormone is transferred to leucocytes during their passage through the thymus; thereafter it is transported exclusively within the leucocytes to points of utilization in the body. A number of relations of the thymus hormone to other endocrine secretions, notably thyroxin and sex hormones, have been stated in some detail but we have made no attempt to repeat those particular studies.

The international situation has made it impossible to secure all of the publications of the Freiburg investigators, but their strong claims to a resolution of problems in which this laboratory has long been interested have led us to this unsuccessful effort to confirm their results.

Preparation of Thymus Extract. Thymus glands of young calves were obtained from a slaughter house where they were frozen immediately after their removal from the animal and were delivered to us within 24 hours. The glands were finely ground while still partially frozen, and the tissue was then extracted in the cold with 5 times its volume of acetone for three 8-hour periods. The acetone was removed under reduced pressure and the residue taken up in ethyl ether. When the ether was distilled off *in vacuo* there remained a relatively large quantity of viscid yellow oil. This product, here called "thymus oil," was subsequently kept at low temperatures; for injection it was either undiluted or diluted with an equal quantity of propylene glycol. Three different extracts were prepared and tested. None of the publications available to us contained a detailed description of the method by which the thymus hormone was extracted, but the apparent simplicity of its preparation and available descriptions of its properties make it wholly probable that our "thymus oil" is identical with or quite similar to the product most commonly used by the Freiburg group.

Results and Discussion. Our first effort to discover physiologic activity in the thymus preparation was directed to total white cell counts in rats following subcutaneous administration of 0.2-0.5 cc of the thymus oil. We here used 2 groups of 6 adult male treated rats together with an equal number of control rats injected with sesame oil. As is to be expected in rats there was considerable variation in the white cell counts from time to time; but there was no indication of a leucocytosis, even within the physiologic range, following ad-

ministration of the thymus oil. The variations in leucocyte count observed by Bomskov[1] following injection of his thymus hormone are well within the normal range for rats. Significant differences within this range are exceedingly difficult to establish, and this would require a much larger number of observations than appears in the published data. It is also notable that the local reaction to the oil, often marked in our animals, is a factor which requires careful control.

In an attempt to observe a "diabetogenic" action of our thymus

TABLE I.

Blood Sugar and Liver Glycogen in Rats and Pigeons Injected Once with Thymus Oil. Time After Injection Usually Is Same as the Period of Fasting.

Test or Control	Dosage, cc	No. of animals	Blood sugar, mg% At hrs			Liver glycogen, mg% At hrs				
			12	17	24	5	12	17	24	36
Male rats aged 3-5 months.										
Thymus oil	.4*	13							.56	
Sesame "	"	9							.59	
Thymus "	.2	16				4.9	0.9		.93	.50
Sesame "	"	16				4.0	2.1		.90	.42
Thymus "	"	4					1.3			
Sesame "	"	4					1.1			
Thymus "	"	4					0.8			
Sesame "	"	4					1.6			
Thymus "	"	5					2.2			
Sesame "	"	5					2.3			
Thymus "	"	4					1.7			
Sesame "	"	4					2.7			
Thymus "	"	3					2.4			
Sesame "	"	4					2.9			
Prop. glycol	"	3					2.6			
Immature Carneau pigeons.										
Thymus oil	.2	9			203				.75	
Thymus "	"†	3			189				.85	
Sesame "	"	10			200				.71	
Thymus "	.4	3	210				4.4			
Sesame "	"	3	208				4.2			
Thymus "	"	3		205				2.8		
Sesame "	"	3		196				2.5		
Thymus "	"	3		194				1.0		
Prop. glycol	"	3		209				0.8		

*Most rats injected twice—at 17 and 10 hours before test; fasted 24 hours.

†Daily injections of 0.2 cc for 4 days preceding test; 3 of control here also injected 4 days.

preparations we administered varying doses at various stages of fasting. From 108 rats and 40 pigeons, treated and control, we obtained the liver glycogen and blood sugar values shown in Table I. In small groups of rats the averages for liver glycogen obtained after 12-hour fasts are more often below than above their control, but the difference seems hardly significant and the values obtained at other stages of fasting are mostly in the opposite direction, though likewise without significance. In pigeons measurable effects of thymus oil were obtained neither on liver glycogen nor on blood sugar.

In additional tests thymus oil did not produce ketonemia (24-hr fast, 10 hr after injection) in either rats or pigeons. From these and other results noted above it is clear that we have been unable to obtain a "diabetogenic" action of lipoid extracts of calf thymus.

With a view to a partial test of possible growth effects, or of influence on various organ weights, we administered moderate or large doses of the thymus oil to 21-day rats, 2-day chicks and to immature pigeons during 3-10 days. These tests were essentially negative; in Moon tests on rats of 21 days the suprarenal weights were unchanged and thymus weights (9 tests) were decreased by nearly 50%. Many years ago Romeis[6] made an extensive study of an acetone extract of the thymus and reported that it has growth-inhibiting properties.

Conclusions. With 3 different preparations of an ether-soluble extract of calf thymus unsuccessful attempts were made to confirm certain results reported by Bomskov and coworkers. No significant leucocytosis was obtained in rats. Short-term tests showed no appreciable effects on bodily growth in young rats, chicks and pigeons. Neither glycogenolytic, glycemic nor ketogenic activity was observed in rather extensive tests on rats and pigeons.

[6] Romeis, B., *Z. Exp. Med.*, 1918, **6**, 101.

13601 P

Effect of Various Androgens on Regenerating Anal Fin of Adult *Platypoecilus maculatus* Females.*

Clifford Grobstein. (Introduced by A. M. Schechtman.)
From the Department of Zoology, Oregon State College, Corvallis, Oregon.

Previous experiments[1, 2] have demonstrated that the regenerating anal fin of adult *P. maculatus* females injected with testosterone propionate forms a clearly male-like structure but fails to form the typical gonopod characteristic of the male. The 3,4,5 complex remains short and differentiation of the various specialized structures within the complex is atypical. In seeking further factors which must be controlled in order experimentally to produce typical gonopod formation in the female, it seemed necessary to determine whether the nature of the morphogenetic agent itself, *i. e.*, of the injected androgen, is of any influence in determining the character of the response. Therefore, equal dosages by weight (2 mg/cc) of androsterone,† dehydroandrosterone, androstenedione, testosterone, methyl testosterone, and ethinyl testosterone have been tested on the regenerating fin and the induced responses compared with one another and with that obtained with testosterone propionate.

The technics employed were in all cases similar to those previously described.[1, 2] The anal fins were cut away across the bases of the rays and intraperitoneal injection was begun on the 9th or 10th day of regeneration. Each animal received 0.05 cc of sesame oil solution containing the appropriate androgen every fifth to seventh day until the response was complete. In most cases a concentration of 2 mg/cc was given. In the case of androsterone, however, several additional groups were run at higher and lower dosages (0.5-5 mg/cc). A total of 67 females were used, 35 receiving androsterone, 10 dehydroandrosterone, 6 ethinyl testosterone, 6 methyl testosterone, 5 testosterone, and 5 androstenedione. Controls received plain sesame oil.

Results. Clear-cut differences in the response of the regenerating anal fin to the various androgens tested have been obtained. An-

* Contribution from the Aquarium, University of California, Los Angeles. I am indebted to Dr. A. W. Bellamy for materials and facilities.

1 Grobstein, C., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 484.

2 Grobstein, C., *J. Exp. Zool.*, 1942, **89**, 305.

† The androgens were generously supplied by Dr. Erwin Schwenk of Schering Corp.

drosterone proved by far the most effective substance in simulating male gonopod formation, although even here a typical gonopod was not produced. In the more favorable cases the 3,4,5 complex was longer than that produced by animals treated with testosterone propionate but nevertheless shorter than that of the characteristic male structure. The specialized ray segments in the distal portion of the complex were remarkably similar to the blade, terminal hook, proximal and distal serrae, etc., of the normal male. The more proximal segments were slightly less typically modified into spoon support, spoon, etc. In general, the response differed from typical gonopod formation chiefly in the incomplete elongation of the third, fourth, and fifth rays.

Methyl and ethinyl testosterone, on the other hand, induced the least typical responses. Injection of the former, in particular, resulted in the production of highly atypical, though clearly masculinized fins. Growth of the 3,4,5 complex was extremely limited and differentiation of the specialized structures within the complex abortive. The final structure produced both by methyl and ethinyl testosterone was decidedly less typical than that previously obtained with testosterone propionate.

The other 3 androgens tested gave responses intermediate to the 2 described. Dehydroandrosterone produced as much, or nearly as much, growth of the 3,4,5 complex as androsterone, but the subsequent differentiation was quite atypical. The testosterone response resembled that of testosterone propionate but proceeded more slowly. Androstenedione gave somewhat variable results, but in general also resembled testosterone propionate in its effect. More data are required before comparisons may be made among the effects of testosterone, testosterone propionate, and androstenedione.

If consideration is limited for the present to 3 substances on which the data are clearest, the order of effectiveness in simulating gonopod formation when administered in concentration of 2 mg/cc is: androsterone, testosterone propionate, methyl testosterone. If the rapidity of response induced by each of these substances be compared, *i. e.*, the interval between beginning of injection and completion of differentiation as judged by reduction of the fleshy cutis,[3] the order is just the reverse. Where the response to methyl testosterone was completed in 13 days, that to testosterone propionate took approximately 30 days, and that to androsterone was complete in only 3 out of 10 animals at 45 days.

[3] Grobstein, C., *Univ. Cal. Publ. Zool.*, 1940, **47**, 1.

13602 P

Restoration of Dorsal Root Potential by Strychnine after Abolition by Partial Sectioning of Spinal Cord.

F. T. Dun. (Introduced by T. P. Feng.)

From the Department of Physiology, Peiping Union Medical College, Peiping.

According to the interpretation of Barron and Matthews[1] the primary site of origin of the spinal cord potential as led electrotonically from the dorsal root is in the dorsal root terminations. Bonnet and Bremer[2] and Eccles,[3] however, think that the dorsal root potential more probably originates in the internuncial neurones. That internuncial neurones can be the origin of the dorsal root potential can be simply demonstrated with the aid of strychnine in the following type of experiment (Fig. 1).

Toads were used. The spinal cord with its roots was exposed and the brain severed from the cord just below the medulla under ether anesthesia, care being taken to preserve the blood supply of the

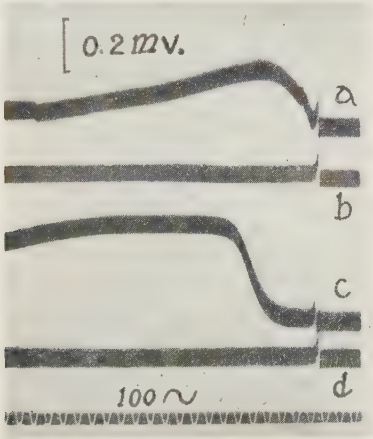


Fig. 1.

To show the restoration of the dorsal root potential by strychnine after abolition by partial sectioning of the spinal cord. Leading electrodes on the right 3rd dorsal root, 0.7 and 3 mm from cord. Stimulating electrodes on the ipsilateral 9th dorsal root, 13 and 16 mm from cord. a, control. b, 5 min. later, after a transverse dorsal cut midway between the two roots, note abolition of the potential. c, Another 33 min. later, after strychnine, note the return of the potential and that it has an abnormally long latency. d, Another 4 min. later, after transection of the whole spinal cord between the roots, note the disappearance of the restored potential. Curves to be read from right to left.

1 Barron, D. H., and Matthews, B. H. C., *J. Physiol.*, 1938, **92**, 276.

2 Bonnet, V., and Bremer, F., *C. R. Soc. Biol., Paris*, 1938, **127**, 806, 812.

3 Eccles, J. C., *Ann. Rev. Physiol.*, 1939, **1**, 363.

cord. Between the discontinuance of the ether and the beginning of the observations there was an interval of at least 40 min. Curare was given to eliminate movements of the animal. The stimulus was applied on the 9th dorsal root while the leading electrodes were on the ipsilateral 3rd root. With the toads used the points of entrance of these two roots into the cord were usually 7-8 mm apart. After ascertaining the presence of the potential and its size, a partial cut of the spinal cord was made midway between the two roots. The cut was sometimes a transverse dorsal one, of a depth corresponding roughly to about ¼ of the thickness of the spinal cord, calculated to sever the fiber tracts in both dorsal columns; sometimes it was limited to the ipsilateral dorsal half of the cord, severing the dorsal column on one side only. Either procedure invariably abolished the potential. A 1/20,000-1/10,000 solution of strychnine nitrate was then irrigated repeatedly on the spinal cord. Within about 10 min. the stimulation of the 9th root was found again to produce large potential in the 3rd root. In about 30 min. the restored potential generally became larger than, and could be 3-4 times as large as the original potential before the cut. As a control against the possibility of the restored potential being due to physical spreading of potential changes from below the cut, the latter was then extended to transect the whole spinal cord; this invariably abolished the potential again. It may also be noted that with a strychninized spinal cord to begin with, such partial cuts as normally abolished the potential, had very little effect.

The dorsal root potential obtained after strychninization presents several interesting and puzzling characteristics. A striking point is that while greatly increased in size it has a much longer latency. The peculiarities of the strychnine potential are now under detailed study. But it seems to be immediately obvious that the restored potential due to strychnine described above must be essentially of internuncial neurone origin.

13603

Rates of Hemolysis in Human Blood Stored in Dextrose Solutions and in Other Mixtures.

Elmer L. DeGowin, John E. Harris and Joy Bell.

From the Departments of Internal Medicine and Obstetrics and Gynecology, State University of Iowa, Iowa City.

One of the chief criteria for determining the suitability of preserved blood for transfusion is the extent of spontaneous hemolysis during storage. The maximal safe period of storage, therefore, depends upon the rate of hemolysis. Long storage is especially desirable in small blood banks and in the adaptation of stored blood for military and civilian emergencies.[1] It has been shown[2, 3] that hemolysis in human blood stored at 2°C begins almost immediately and proceeds at varying rates depending upon the preservative used. This paper presents data to show that the Rous-Turner dextrose-citrate mixture as modified by DeGowin, Harris, and Plass[2] is particularly adapted to long storage of human blood.

In these experiments, blood was drawn into appropriate volumes of 3.2% sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in water, the exact dilution being estimated by the hematocrit method of Van Allen. Under aseptic technic, other solutions were added in accurate proportions and the resultant mixtures apportioned into cotton-stoppered flasks and stored at 2°C. At suitable intervals, flasks from each series were removed from storage and the plasmas separated by centrifugation. The plasma hemoglobin was determined by the Wu method[4] and the percentage of hemolyzed erythrocytes was calculated from the plasma hemoglobin value and the hematocrit reading.

Table I contains the data from representative experiments. During the first 5 days of storage hemolysis was minimal in all mixtures, but beyond that point there was considerable variation. Dilution with electrolytes (NaCl, KCl) provoked the most rapid hemolysis, and blood-citrate hemolyzed nearly as fast. Dilution with isotonic dextrose and sucrose solutions greatly inhibited hemolysis, dextrose being more efficient than sucrose. The blood-dextrose-citrate mix-

1 DeGowin, E. L., and Hardin, R. C., *War Medicine*, 1941, **1**, 326.

2 DeGowin, E. L., Harris, J. E., and Plass, E. D., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 126.

3 DeGowin, E. L., Harris, J. E., and Plass, E. D., *J. Am. Med. Assn.*, 1940, **114**, 850.

4 Wu, H., *J. Biochem.*, 1922, **2**, 189.

TABLE I.
Rates of Hemolysis in Human Blood Stored at 2°C in Various Preservative Mixtures. Values Expressed as Percent of Erythrocytes Hemolyzed.

Experiment No.	20A	18A	20B*	22A*	18C	20D	21A	20C	21B
Blood Mixture (vol.)									
Blood	23	23	10	10	10	10	10	10	10
Sodium Citrate 3.2%	2	2	2	2	2	2	2	2	2
Dextrose 5.4%	—	—	13	13	—	—	—	13	—
Sucrose 10%	—	—	—	—	13	—	—	—	—
Sucrose 4.5%	—	—	—	—	—	13	—	—	—
NaCl 0.9%	—	—	—	—	—	—	13	—	—
NaCl 0.5%	—	—	—	—	—	—	—	sol	—
KCl 1.15%	—	—	—	—	—	—	—	—	13
Percent Hemolysis									
Days storage									
0	0	0	0	0	0	0	0	0	0
5	0.09	0.17	—	.03	.03	0.03	—	.05	—
10	—	0.73	—	.08	.10	—	—	—	—
15	1.51	—	.08	.09	.22	1.14	2.64	.29	1.92
20	3.58	3.24	—	—	.44	1.01	—	—	—
25	7.20	—	.38	.21	.60	—	21.80	.62	19.10
30	13.80	9.08	—	.27	.85	2.57	—	—	—
35	—	—	.47	.43	—	—	—	.91	—
40	—	—	—	.92	—	6.75	—	—	—
45	—	—	.63	—	—	—	—	2.05	—

*Modified Rous-Turner mixture (DeGowin, Harris, and Plass).
sol—Dextrose dissolved in NaCl solution instead of distilled water. Otherwise sugars were dissolved in distilled water.

ture of DeGowin, Harris and Plass resulted in only 2-4% as much hemolysis in 30 days as did blood-citrate. In blood-dextrose-citrate there was less than ½ as much hemolysis in 30 days as occurred in blood-citrate in 10 days. These observations confirm the qualitative studies of Rous and Turner.[5]

The superiority of preservative mixtures containing dextrose has now been widely recognized. Many modifications of the Rous-Turner mixture[6-9] have been devised to reduce the final volume of the blood mixture by decreasing the volume of isotonic dextrose solution or by using hypertonic concentrations of the sugar. In each case the resulting dextrose concentration has been less than the approximate 3% recommended by Rous and Turner and by DeGowin, Harris, and Plass.

Table II presents data upon the effect of varying concentrations of dextrose (1.0-6.0%) on the rate of hemolysis during storage, when

[5] Rous, Peyton, and Turner, J. R., *J. Exp. Med.*, 1916, **23**, 219.

[6] Gwynn, C. A., and Alsever, J. B., *Am. J. Med. Sc.*, 1939, **198**, 634; Alsever, J. B., and Ainslie, R. B., *N. Y. State J. Med.*, 1941, **41**, 126.

[7] Maizels, M., and Whittaker, N., *Lancet*, 1940, **1**, 590.

[8] Aylward, F. X., Mainwaring, B. R. S., and Wilkinson, J. F., *Lancet*, 1940, **1**, 685.

[9] Barton, F. E., *N. England J. Med.*, 1941, **225**, 176.

the sodium citrate content was kept constant. Reduction of the final concentration of dextrose in the blood mixture below 3% resulted in increasing the hemolysis in 30 days of storage. Utilization of dextrose solutions of stronger than 10% did likewise. Other difficulties encountered in the use of hypertonic dextrose solutions are discussed elsewhere.

Summary. The addition of dextrose to blood-citrate mixtures markedly inhibits hemolysis during storage at 2°C. Sucrose is not so effective and NaCl and KCl increase the rate of hemolysis. The final concentration of dextrose in the blood mixture required for the maximal inhibition of hemolysis is approximately 3%. The use of a preservative mixture containing this amount of dextrose ordinarily permits the safe storage of blood for 30 days or more.

TABLE II.

Hemolysis in Blood-Dextrose-Citrate Mixtures After Storage for 30 Days at 2°C. Concentration of Dextrose Was Adjusted by Varying Volume and Concentration of Sugar Solutions. Concentration of Sodium Citrate Remained Constant at 0.26% (with exception of 19A1 and 19B1).

Experiment	Human blood (vol.)‡	Dextrose sol. added (vol.)	Dextrose sol. added (conc., %)	3.2% sodium citrate (vol.)	Dextrose conc. in mixture, %	Erythrocytes hemolyzed in 30 days (2 exper.) %
19B1	40	66.4	10.0	26.4	5.7	.29, .40
19B2	40	52.0	10.0	8.0	6.0	.25, .32
19B3	40	40.0	10.0	6.8	5.5	.25, .43
19B4	40	30.0	10.0	6.0	4.7	.28, .24
19B5	40	20.0	10.0	4.8	3.6	.33, .41
19B6	40	10.0	10.0	4.0	2.1	.64, 1.42
19A1*	40	66.4	5.4	26.4	3.0	.22, .27
19A2†	40	52.0	5.4	8.0	3.2	.20, .43
19A3	40	40.0	5.4	6.8	2.9	.31, .92
19A4	40	30.0	5.4	6.0	2.4	.47, .50
19A5	40	20.0	5.4	4.8	1.8	.97, 1.18
19A6	40	10.0	5.4	4.0	1.3	1.78, 2.54
23A1	35.7	10.7	23.3	3.5	5.0	.64, .58
23A2	35.7	10.7	18.6	3.5	4.0	.58, .41
23A3	35.7	10.7	14.0	3.5	3.0	.83, .49
23A4	35.7	10.7	9.3	3.5	2.0	1.87, .93
23A5	35.7	10.7	4.6	3.5	1.0	6.69, 2.70
23B1†	43	56.0	5.5	7.8	2.9	.26, .21
23B2	43	44.0	6.3	6.6	3.0	.28, .25
23B3	43	36.0	6.9	6.0	2.9	.32, .25
23B4	43	27.0	8.0	5.4	3.0	.40, .26
23B5	43	20.0	9.9	4.8	3.0	.45, .24
23B6	43	13.0	13.9	4.2	3.0	.82

*Rous-Turner mixture.

†Modified Rous-Turner mixture (DeGowin, Harris, and Plass).

‡Under the conditions of collecting blood the volume of the blood could only be approximated. This accounts for the disparity between calculated final concentrations of dextrose and actual values which are given in the 6th column.

13604

Osmotic Changes in Erythrocytes of Human Blood During Storage.

Elmer L. DeGowin, John E. Harris, Joy Bell and Robert C. Hardin.

From the Departments of Internal Medicine and Obstetrics and Gynecology, State University of Iowa, Iowa City.

Scant attention has been given to osmotic changes occurring in erythrocytes stored at low temperatures although most workers insist upon the use of isotonic solutions in the preservative fluid. Rous and Turner[1] found no relationship between the resistance of red cells to hypotonic saline solutions and to mechanical shaking. Several investigators[2-5] have reported increased fragility of the erythrocytes during storage. Maizels and Whittaker[6] noted that although blood stored in hypertonic saline solutions showed little hemolysis the erythrocytes were ruptured when placed in normal plasma because their contents had become equilibrated to the hypertonic plasma. The permeability of red cells to dextrose suggested the possibility of a similar reaction when that sugar is used in the preservative. The present study is concerned with certain phases of this problem.

Table I presents the data from 3 representative experiments. Changes in cell volume were calculated from hematocrit readings. The fragility of the erythrocytes was estimated by adding 0.1 cc of whole blood mixture to 2.0 cc of each of a series of sodium chloride solutions with concentrations ranging from 0.24% to 1.20% in increments of 0.04%. After 1 hour at room temperature, the highest concentrations showing initial and complete hemolysis were noted—values are expressed in that order. For testing washed cells the plasma was removed after centrifugation and the cells washed twice with 0.95% sodium chloride solution before being added to the series of saline solutions.

In citrate-blood there was a progressive increase in cell volume during storage which was approximately paralleled by increase in fragility of both washed and unwashed cells. In dextrose-citrate-

1 Rous, Peyton, and Turner, J. R., *J. Exp. Med.*, 1916, **23**, 219.

2 Doepp, M., *Deutsch. Z. f. Chir.*, 1934, **243**, 736.

3 Belk, W. P., Henry, N. W., and Rosenstein, F., *Am. J. Med. Sc.*, 1939, **198**, 631.

4 Kolmer, J. A., and Howard, Mary, *Am. J. Med. Sc.*, 1940, **200**, 311.

5 Muether, R. O., and Andrews, K. R., *Am. J. Clin. Pathol.*, 1941, **11**, 314.

6 Maizels, M., and Whittaker, N., *Lancet*, 1940, **1**, 590.

TABLE I.
Changes in Cell Volume and Fragility of Human Blood Stored at 2°C in Various Preservative Mixtures.

		Days of storage at 2°C						
		0	5	10	15	20	25	30
Blood + Citrate 20A								
% change in cell volume		0	0		+13.8	+21.4	+31.5	+47.0
Fragility	Unwashed	.40-.32	.64-.32		.64-.32	.88-.40	.88-.40	.92-.44
	Washed	.40-.32	.64-.36		.64-.36	.88-.40	.88-.40	.92-.44
Blood + Dextrose + Citrate 22A								
% change in cell vol.		+53.0	+34.1	+38.2	+41.0	+43.0		+50.0
Fragility	Unwashed	.80-.64	.76-.56	.76-.56	.80-.60	.84-.60	.80-.64	.84-.64
	Washed	.44-.32	.36-.24	.36-.24	.36-.24	.36-.24	.40-.28	.36-.28
Blood + Sucrose + Citrate 20D								
% change in cell vol.		+21.4	—4.5		—5.9	—9.2		—5.2
Fragility	Unwashed	.40-.28	.36-.24		.44-.24	.52-.24		.56-.24
	Washed	.40-.32	.40-.24		.48-.24	.52-.24		.64-.24

20A = 23 volumes blood + 2 volumes 3.2% sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in water.
22A = 10 volumes blood + 2 volumes 3.2% sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in water + 13 volumes 5.4% dextrose in water.
20D = 10 volumes blood + 2 volumes 3.2% sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in water + 13 volumes 4.5% sucrose in water.

blood[7] the changes were more complicated. Within 1 hour after the addition of dextrose the cell volume had increased over 50%. During the first 5 days of storage the swelling diminished slightly but again gradually approached a maximum in 30 days. These changes were reflected in the high fragility for unwashed cells. Washed cells, however, showed normal fragility indicating that the swelling was due to the diffusion of dextrose and water into the erythrocytes and that this process was reversible.

Sucrose was used in hypotonic concentration since previous observation had shown that the cells shrank in 10% solution. In the recorded experiment with sucrose there was an initial increase in cell volume followed within 5 days by persistent shrinkage. The fragility of both washed and unwashed cells was well correlated with volume changes. The rapid change during the first 5 days was associated with marked loss of cellular potassium.

There was no direct correlation between the amount of cellular swelling and the extent of hemolysis during storage.[7] Dextrose-citrate-blood showed the greatest swelling and the least hemolysis while sucrose-citrate-blood underwent moderate hemolysis with shrunken cells. Citrate-blood, showing slightly less swelling than dextrose-citrate-blood, was greatly hemolyzed after 30 days.

[7] DeGowin, E. L., Harris, J. E., and Bell, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 481.

It has been shown previously[7] that a final concentration of approximately 3% dextrose is optimal for the inhibition of hemolysis during storage but the total volume of the modified Rous-Turner mixture has militated against its wide acceptance. Table II presents the changes in cell volume and in fragility of blood stored in various concentrations of dextrose. Hypertonic dextrose concentrations induced less swelling in the erythrocytes during storage than did more nearly isotonic solutions. The cell contents became equilibrated to the hypertonic plasma so that the cells ruptured when placed in isotonic saline. Washing the cells in isotonic saline, however, resulted in normal fragility values for the cells which did not rupture, indicating that the diffusion of dextrose and water was responsible for the hypertonicity originally observed.

The gross appearance of blood stored in hypertonic dextrose is excellent but it is dangerous to ignore the hypertonicity of the erythrocytes. Serious intravascular hemolysis may result when such blood is transfused. Of 128 stored bloods (13 volumes of 10% dextrose, 2 volumes of 3.2% sodium citrate, and 10 volumes of

TABLE II.

Changes in Cell Volume and Fragility of Human Blood Stored at 2°C for 30 Days in Various Concentrations of Dextrose. The Concentration of Sodium Citrate Was Uniformly 0.26%.

Experiment	Blood (vol.)	Dextrose sol. added (vol.)	Dextrose sol. added (conc., %)	Dextrose conc. in mixture, %	Values after 30 days (2 experiments): Change in cell vol., %	Values after 30 days (2 experiments): Fragility of unwashed cells
23A1	35.7	10.7	23.3	5	+14.6	1.20-.72
					+11.1	1.20-.68
23A2	35.7	10.7	18.6	4	+17.6	1.10-.72
					+20.9	1.20-.72
23A3	35.7	10.7	14.0	3	+23.6	1.10-.68
					+29.1	1.10-.64
23A4	35.7	10.7	9.3	2	+30.0	.96-.68
					+40.5	1.00-.56
23A5	35.7	10.7	4.6	1	+32.0	.96-
					+44.3	.88-.48
23B1*	43	56	5.5	2.9	+40.6	.80-
					+28.6	.80-.52
23B2	43	44	6.3	3	+26.9	.84-.68
					+28.8	.80-.52
23B3	43	36	6.9	2.9	+28.2	.88-.68
					+24.8	.84-.52
23B4	43	27	8.0	3	+23.1	.88-.68
					+25.3	.88-.52
23B5	43	20	9.9	3	+19.4	.96-.68
					+ 0.3	1.00-.60
23B6	43	13	13.9	3	+33.8	1.10-.68

*Modified Rous-Turner mixture (DeGowin, Harris, and Plass).

blood), 16 produced post-transfusion hemoglobinuria (one specimen had been stored only 3 days), 27 were discarded because the cells hemolyzed in 0.9% saline, and 85 were transfused without detectable hemoglobinuria. Whenever unwashed cells were hemolyzed by 0.9% saline they were also ruptured in fresh human plasma. Incompatibility from isohemagglutinins and isohemolysins was excluded in the cases studied. Evidently the osmotic activity of stored red cells must be considered in determining the suitability of preserved blood for transfusion.

It has been observed[8] that hemolysis frequently occurs after a few hours' refrigeration when large quantities of blood (500 cc) are introduced into warm (room temperature) dextrose-citrate mixture (modified Rous-Turner) but that effect is avoided if the mixture is ice-cold. Early hemolysis, moreover, does not occur when small quantities of the blood mixture are employed, a fact which suggests that the rate of cooling is important.

To test this hypothesis, blood was drawn into flasks of dextrose-citrate (modified Rous-Turner) at room temperature (27°C). After 2, 60, 120, and 210 minutes the erythrocyte fragility was determined and specimens were quickly chilled to 0°C. Initial hemolysis occurred at 0.44%, 0.76%, 0.84%, and 0.88% sodium chloride solution, respectively. Specimens which were chilled after 2 hours or more at room temperature showed considerable hemolysis, whereas the controls kept at room temperature did not hemolyze. Apparently, dextrose and water diffused into the cells at room temperature and the reduction in temperature ruptured the swollen erythrocytes. Jacobs, Glassman, and Parpart[9] report that as the temperature of an erythrocyte suspension is reduced, initial hemolysis occurs in progressively higher concentrations of hypotonic saline solution. The mechanism by which the quick chilling arrests the diffusion of dextrose and water into the cells is obscure but the empirical fact is important in the collection of dextrose-citrate-blood (modified Rous-Turner) for transfusion.

Summary. In citrate-blood the erythrocytes undergo progressive swelling during storage at 2°C; this is reflected in the increase in the fragility to hypotonic saline solutions. This property is not reversible by washing in isotonic saline. Red cells stored in sucrose-citrate solutions shrink. Erythrocytes in 5.4% dextrose-citrate

[8] DeGowin, E. L., Harris, J. E., and Plass, E. D., *J. Am. Med. Assn.*, 1940, **114**, 850.

[9] Jacobs, M. H., Glassman, N. H., and Parpart, A. K., *J. Cell. and Comp. Physiol.*, 1931, **60**, 95.

undergo rapid swelling which is due to the diffusion of dextrose and water into the cells. The process can be reversed by washing with isotonic saline. The addition of hypertonic dextrose solutions to blood-citrate produces hypertonic cell contents during storage. The transfusion of such cells results in intravascular hemolysis by action of the recipient's plasma. The osmotic activity of the erythrocytes thus becomes another criterion for the suitability of stored blood for transfusion. Blood collected in dextrose solutions should be chilled rapidly to avoid osmotic hemolysis during refrigeration.

13605

Study with Radioactive Isotopes of Excretion of Calcium and Strontium by Way of the Bile.*

David M. Greenberg and Frances M. Troescher.

From the Division of Biochemistry, University of California Medical School, Berkeley.

The significance of the bile as a pathway for the excretion of calcium has attracted attention because of the relatively high calcium concentration of gall bladder bile.[1] In experiments on 2 bile fistula dogs, Gillert[2] found that the calcium in the bile was about ⅔ the amount excreted in the urine.

Tracer studies with radioactive calcium and strontium offer a method of obtaining information on the excretion of these ions by way of the bile that simplifies many of the difficulties usually present in this type of experiment, such as the factor of reabsorption from the gall bladder[3] and the source of the calcium in the bile. Through the use of labeled ions, it is possible to determine the fraction of an administered dose of calcium or strontium that appears in the bile. The results of such experiments on bile fistula rats are described in this communication.

Methods. Rats weighing between 400 and 500 g, with an artificial gall bladder type fistula, prepared according to the operation of

* Aided by a grant from the Christine Breon Fund of the University of California Medical School.

1 Drury, D. R., *J. Exp. Med.*, 1924, **40**, 797.

2 Gillert, E., *Z. ges. Exp. Med.*, 1924, **43**, 539.

3 Johnston, C. G., Ravdin, I. S., Austin, J. H., and Morrison, J. L., *Am. J. Physiol.*, 1932, **99**, 648.

Sawyer and Lepkovsky[4] as modified by Harrington, Greaves and Schmidt,[5] were used in the experiments. After the operation, a saline-glucose solution containing dissolved bile, was administered to the animals by stomach tube at about 6-hour intervals, immediately after the collection of each bile sample from the artificial glass gall bladder. The radioactive elements as the lactate salts were injected intraperitoneally in single doses containing 3.4 mg of calcium or 2 mg of strontium. The radioactivity of the strontium samples (Sr^{89}) was measured on a Lauritzen electroscope and of the calcium samples on a Geiger-Müller counter. A bell type of counter tube† with a mica window was used to detect the soft radiation emitted by the Ca^{145}. Standards were prepared by adding suitable aliquot portions of the radioactive calcium or strontium lactate samples to bile collections obtained prior to the initiation of the experiments.

Results. The results of the experiments are shown graphically in Figs. 1 and 2. Fig. 1 shows the curves for the total of the amount of Ca*‡ and Sr* eliminated in the bile. The greatest rate of elimination of calcium or strontium by the bile occurs during the first 2 days. Thereafter, the amount falls off to very small values. The curves for calcium and strontium are nearly similar. Calcium shows

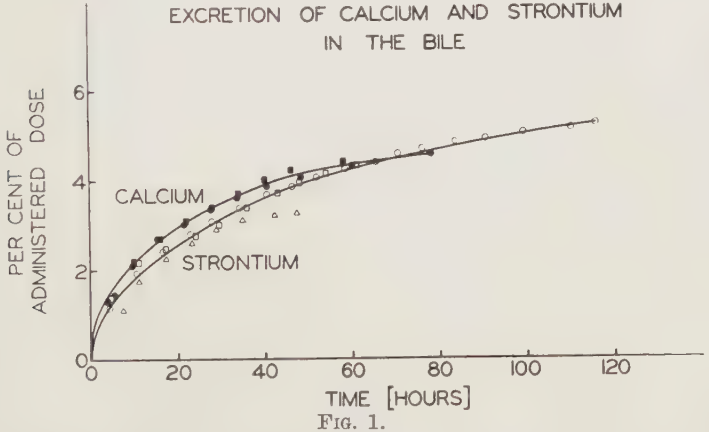


FIG. 1.

The rate of elimination of Ca* and Sr* in the bile. Full symbols represent individual calcium values, open symbols, strontium values.

4 Sawyer, L., and Lepkovsky, S., *J. Lab. Clin. Med.*, 1935, **20**, 958.

5 Harrington, F. G., Greaves, J. D., and Schmidt, C. L. A., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 611.

† Developed by Dr. Dodson of the California Institute of Technology. Unpublished.

‡ Chemical symbol with asterisk is used to represent element labeled with radioactive isotope.

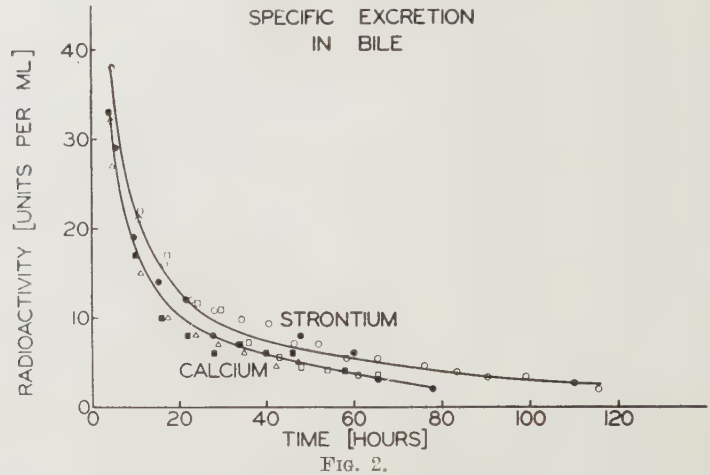


FIG. 2.

Specific excretion of Ca* and Sr* in the bile. The unit of specific activity (radioactivity per ml) is 1/10,000 part of injected dose. Full symbols represent individual calcium values, open symbols, strontium values.

a slightly faster rate of elimination initially, but the curve for strontium overtakes that for calcium in about 60 hours.

The total excreta was analyzed and yielded the following results: In 65 hours the Sr* in the excreta was 13.4, 22.0, and 25% respectively of the administered dose or an average of about 20%. In the same interval of time, there was an average elimination of about 4.5% of the Sr* in the bile. Thus the bile was the pathway for the elimination of about 20% of the injected Sr*. In the experiments with calcium, at the end of 60 hours, the excreta contained 7.3 and 19% respectively of the Ca*, while the amount in the bile was 4.5 and 4.4% respectively of the administered dose.

If the bile secreted by the fistula animals is even a rough measure of the normal bile output, then the present data indicate that the bile is a pathway of considerable importance for the excretion of the alkali earth elements.

The change in specific activity (radioactivity per ml) of the bile with the lapse of time after administration of the Sr* or Ca* is plotted in Fig. 2. Specific activities are represented in terms of a unit which is 1/10,000 part of the dose administered. The curve for the decrease in specific activity appears to approximate a parabola. The drop in the activity of Ca* is somewhat steeper than of Sr* although the curves for the two elements do not differ greatly. In the first sampling period the specific activity is over 0.3%. After 2 or 3 days it drops down to a few hundredths percent.

Summary. Between 4 and 5% of an injected dose of labeled

strontium or calcium is eliminated with the bile out of a total excretion of about 25% in bile fistula animals in a period of about 3 days.

We are greatly indebted to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for the supply of radioactive calcium and strontium. We also are greatly indebted to Miss L. Morse for performing the surgical operations and to D. H. Copp and M. Murayama for valuable assistance.

13606

Radiation Effects on Nervous System and Roentgen-Pigmentation of Goldfish (*Carassius auratus*).

CHARLES DAVISON AND FRIEDRICH ELLINGER.*

From the Neuropathological Laboratory and the Radiotherapy Department, Montefiore Hospital for Chronic Diseases, New York City.

A variety of stimuli has been employed for the study of the relationship between the nervous system and the chromatophores in fish. Smith[1] was the first to produce an eruption of corial melanophores in the goldfish. During studies concerning the suitability of the goldfish for problems of experimental radiation therapy,[2, 3, 4] one of the authors (Ellinger) made some observations on the roentgen pigmentation of goldfish.[5] This led to the assumption of a disturbance in the chromatomotoric nerve center in the medulla oblongata as a possible cause for the observed roentgen pigmentation. This pigmentation started on the head of the fish and spread caudally. To substantiate this assumption, a histopathologic study of the central nervous system of 10 goldfish (8 of which were irradiated) has been made.

Methods. The radiation factors were: 200 kv, 30 ma (mechanical rectification); filter (a) zero, H.V.L. 6 mm Al, corresponding to 0.233 mm Cu (intensity, 230-172 r/min); (b) 0.5 mm Cu and 1 mm Al, H.V.L. 1 mm Cu (intensity, 48 r/min). The distance was

* Aided by a grant from the Emergency Committee in Aid of Displaced Foreign Physicians.

1 Smith, G. M., *Am. J. Cancer*, 1932, **16**, 863.

2 Ellinger, F., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 527.

3 Ellinger, F., *Radiology*, 1940, **35**, 563.

4 Ellinger, F., and Gross, R., *Radiology*, 1941, **37**, 717.

5 Ellinger, F., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 148.

always 50 cm (target surface of water), and the field size 15 x 15 cm. The doses, ranging from 500 to 10,000 r (measured in air), have been given in one session. For each exposure 12 fish were placed in an open Petri dish, 15 cm in diameter, covered with a single layer of gauze fixed by an elastic. For further details of the technic of irradiation the reader is referred to a previous paper.[3] All the brains of those animals were embedded in celloidin, sectioned and stained by the myelin sheath and cresyl violet methods.

Results. The histopathological examination in all instances revealed most pronounced pathological changes in the medulla oblongata of the irradiated central nervous system. Three typical protocols will suffice to describe the findings:

I. *Protocol No. 883.* Goldfish 59A irradiated with 1500 r/air, (minimal lethal dose[3]) developed pigmentation on the 11th day and the fish was killed on the 15th day following irradiation.

Microscopic Examination. In the myelin sheath preparation, some of the myelin fibers in the diencephalon, but especially those of the medulla oblongata, disclosed slight destruction. At the periphery of the medulla oblongata these had a slight spongious appearance (Fig. 1B). Swelling and destruction of myelin fibers were detected (Fig. 1B). A few of the axis cylinders were slightly swollen, had a corkscrew appearance and bulbous terminations. In the cresyl violet preparation, in the areas of demyelinization, there was a slight increase in the glia nuclei (Fig. 2A) and some extravasation of the red blood cells. The nerve cells of the medulla oblongata were diminished in number and most of these were vacuolated (Fig. 2A) or appeared as shadow cells. Complete disintegration of nerve cells was also noted (Fig. 2A). There were a few agonal hemorrhages around the central canal and ventricles.

The spinal cord disclosed a slight swelling and fragmentation of the myelin sheaths especially at the periphery. In the cresyl violet preparation, the anterior horn cells showed vacuolization (Fig. 2B) or complete destruction.

II. *Protocol No. 879.* Goldfish 49A, irradiated with 1000 r/air (sublethal dose[3]). Pigmentation appeared on the 17th day and the fish was killed on the 28th day after irradiation.

Microscopic Examination. The vegetative nerve cells of the medulla oblongata were slightly decreased in number but rich in iron pigment. Some of these, however, showed minor changes, such as shrinkage, occasional chromatolysis and shadow-like appearance. There was no marked increase in the glia nuclei and no extensive proliferation of the vessels. In other sections throughout the central

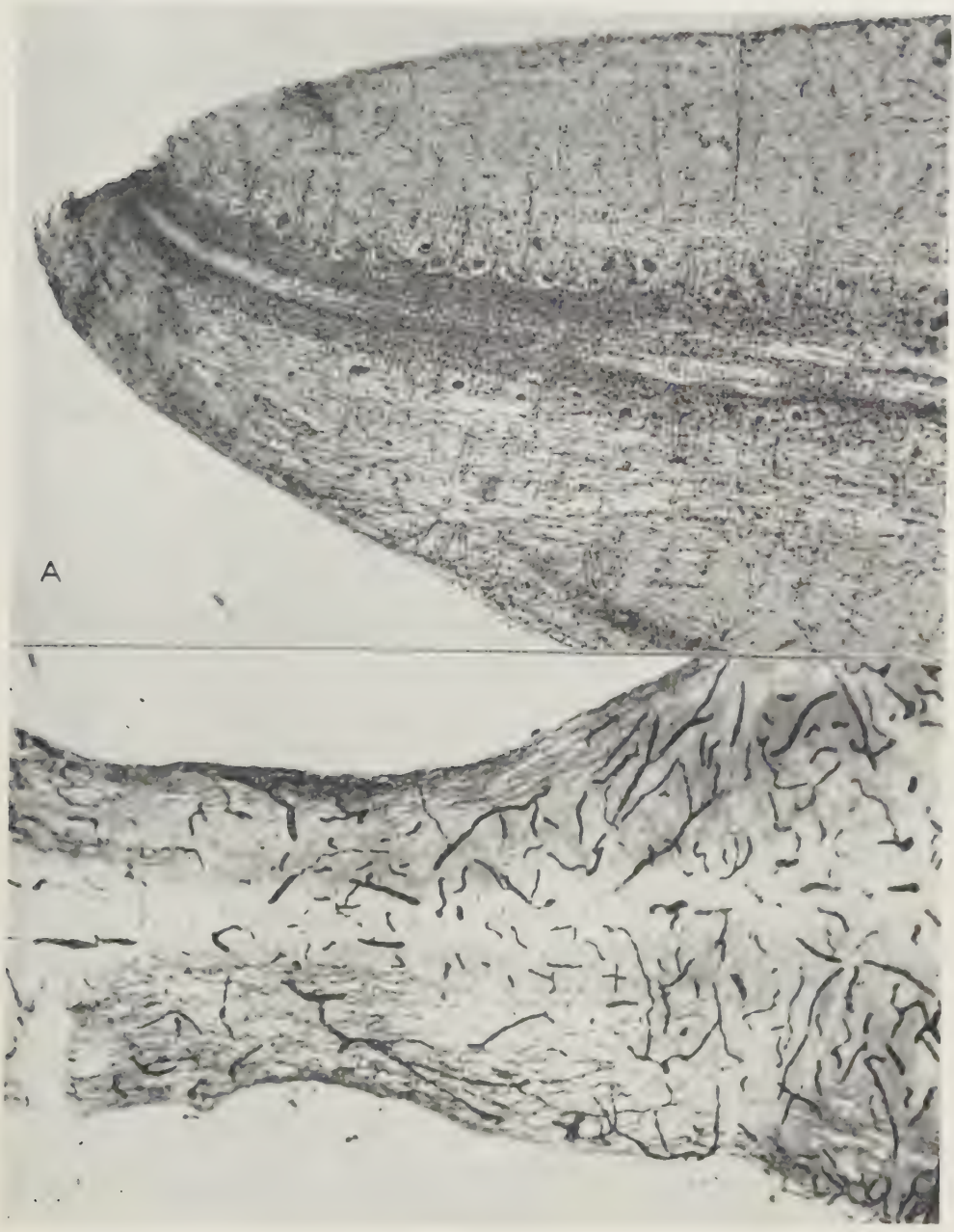


FIG. 1A.

Medulla oblongata of an unirradiated fish. Notice the normal number of nerve cells of the medulla oblongata.

FIG. 1B (Bottom).

Medulla oblongata of a fish irradiated with 1500 r. Note: Slight status spongiosus, swelling and destruction of the myelin sheaths at the periphery of the medulla. Disappearance of the characteristic nerve cells. Myelin sheath, × 100.

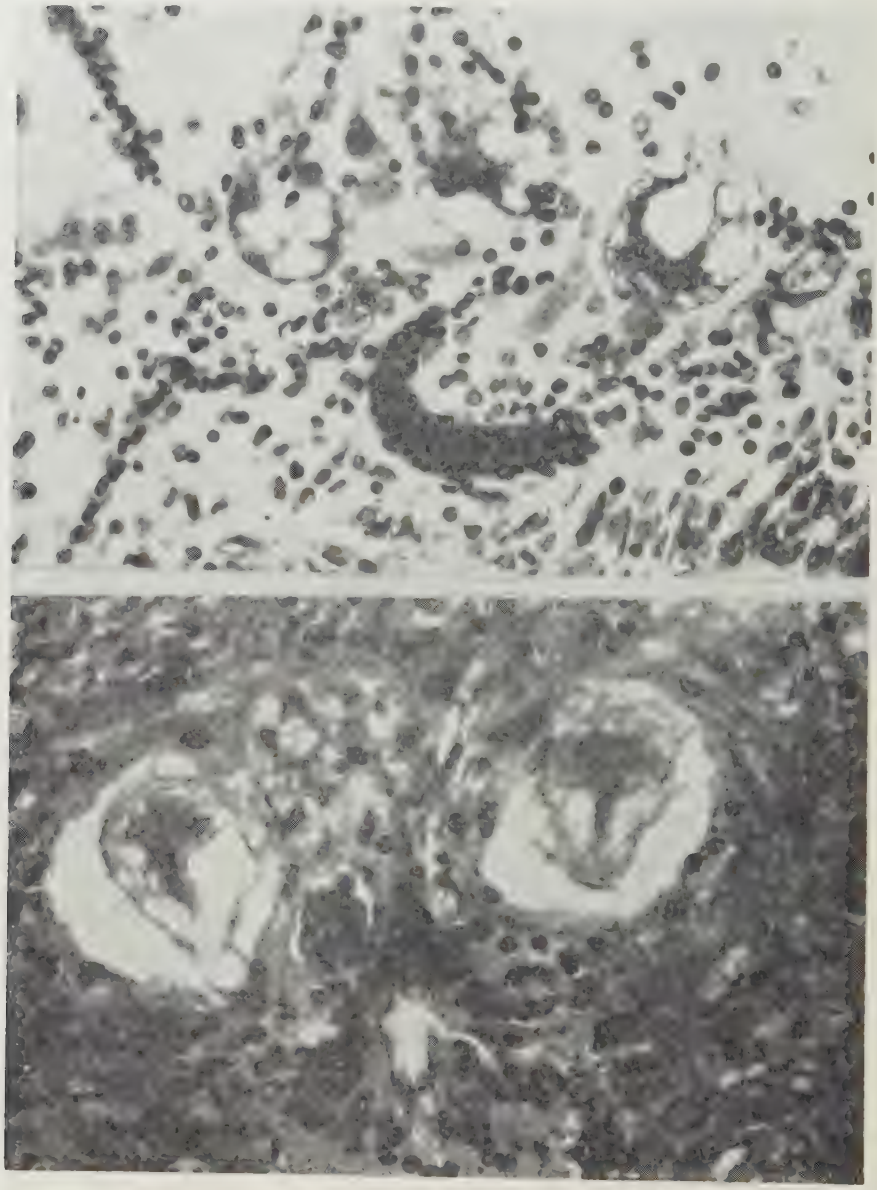


FIG. 2A. (Top).
Medulla oblongata of a fish irradiated with 1500r. Severe cell changes in the area of demyelinization: Vacuolization of nerve cells, increase in glia nuclei and congestion of blood vessels.

FIG. 2B (Bottom).
Spinal cord of a fish irradiated with 1500r. Two large vacuolated nerve cells of the anterior horns. Cresyl violet, × 480.

nervous system, occasional minor changes were found in some of the nerve cells. In one area of the archi cortex, there was some slight proliferation of the vessels.

III. *Protocol No. 848.* Goldfish 42A, irradiated with 800 r/air (minimal dose to produce pigmentation[5]). Slight pigmentation appeared on the 21st day and the fish was killed on the 50th day after irradiation.

Microscopic Examination. No abnormalities were noted in sections stained with the cresyl violet method. In the sections stained by the myelin sheath method there was found a slight demyelinization of the fiber tracts at the periphery of the medulla oblongata.

Summary and Conclusions. Histological examination of the central nervous system of irradiated goldfish revealed: (1) Pronounced pathological changes in all instances, predominantly in the medulla oblongata and extending into the anterior horns of the spinal cord. (2) Alterations degree depending on the dose of roentgen rays. (3) Changes similar in character to those observed in the central nervous system of mammals. These consisted chiefly in demyelinization of fiber tracts, destruction of ganglion cells and a more or less pronounced gliosis.

The fact that these changes are most pronounced in the medulla oblongata, where the chromatomotoric nerve center is located, seems to support the assumption that destruction of this center may be the cause of roentgen pigmentation in the goldfish. In the light of these observations the caudal spread of melanophores can easily be understood as a descending degeneration of the pigmento-motoric nerve fibers. This seems to agree with the experiments of Von Frisch,[6] who produced in another fish (*Phoxinus laevis* L) pigmentation throughout the entire body after mechanical destruction of the pigmento-motoric center in the medulla oblongata. These observations, besides throwing some more light on the interesting interrelationship between central nervous system and pigmentation in fish, seem to be important as a further evidence for the equality in the effects of rays and mechanical stimuli on living matter.[7, 8] Furthermore, the similarity of the character of the histological changes in the central nervous system after irradiation in goldfish and mammals seems to emphasize the suitability of the goldfish for purposes of experimental radiation therapy.

---

[6] von Frisch, K., *Arch. f. ges. Physiol.*, 1911, **138**, 319.

[7] Ellinger, F., *The Biologic Fundamentals of Radiation Therapy*, Elsevier Publishing Co., New York, 1941.

[8] Ellinger, F., The Problem of Recovery from Radiation Effects, appears in *Radiology*.

13607

Rôle of Lipophilia in the Etiology of Obesity.*

Malcolm Block. (Introduced by L. H. Newburgh.)

From the Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Mich.

Bergmann[1] suggested that the adipose cells of the obese possess a quality which permits them to seize an abnormally large amount of circulating fat and to hold this fat in the storage depots when the normal individual would release it as a source of energy. By this mechanism neither ingested nor stored fat is available for energy in the obese individual, thus rationalizing the increased food intake.

The only experimental data to support this concept has been supplied by Hetenyi,[2] who found that the total blood lipids of obese subjects decreased during undernutrition, but remained constant in individuals of normal weight. Hetenyi interpreted this fall in total blood lipids as indicating that the fat could not be mobilized for heat production. This was felt to be direct evidence that lipophilia actually is a determining factor in causing obesity.

Since in each instance Hetenyi's subject lost at least one kilo of body weight during the period of undernutrition, his interpretation could have been readily evaluated by nitrogen balance studies in obese individuals during undernutrition, but no such data were published. It is well known that the glycogen stores in the animal organism are rapidly depleted during 2 to 3 days of undernutrition so that after this preliminary period there remains only fat and protein in the body to be utilized for heat production. If fat stores can not be mobilized and so utilized as is postulated by the theory of lipophilia, a negative nitrogen balance should rapidly ensue, or weight should remain constant. Even though Strang[3] previously demonstrated that obese individuals remain in nitrogen balance during severe undernutrition as long as the protein intake remains one gram per kilo of ideal body weight or higher, nevertheless it was felt that similar nitrogen balance studies over a comparatively long period of

* This study was assisted by a special grant to L. H. Newburgh from the Horace H. Rackham Endowment Fund.

1 v. Bergmann, G., *Oppenheimer Handbuch der Biochemie*, 1910, **4** (Teil 2), 212.

2 Hetenyi, Geza, *Deutsch. Arch. f. klin. Med.*, 1936, **179**, 134.

3 Strang, J. M., McClugage, H. B., and Evans, F. A., *Am. J. Med. Sc.*, 1931, **181**, 336.

time in conjunction with the determination of total blood lipids would help in evaluating the theory of lipophilia.

Procedure. Three obese female subjects and 3 female controls of normal weight were given constant daily diets of known nitrogen content. Total daily protein intake was always higher than one gram per kilo of ideal body weight. The initial caloric value of the diet was approximately 80% of the basal requirement for the individual. This was decreased 20% at weekly intervals so that by the fourth week the caloric value of the diet was only 20% of the basal requirement. Daily 24-hour urinary nitrogen excretion was determined by the macro-Kjeldahl method and total post-absorptive blood serum lipids were determined by the micro-oxidative procedure of Boyd[4] at weekly intervals. Total heat production of each obese individual was measured by the determination of insensible loss of weight[5] on at least 6 successive days and the average of all daily values was assumed to be the average daily heat production of the individual for the entire experimental period.

Results and Discussion. Table I shows the data for the obese subjects. In each instance (with one exception) positive nitrogen balance was maintained throughout the entire period of observation. Blood lipids varied considerably with some tendency to rise during the early days of undernutrition but to fall later to nearly their initial values. In the control subjects (Table II) there was some tendency for a negative nitrogen balance to occur, but the blood lipids varied essentially in the same direction as for the obese subjects. Thus from our data we could find no evidence that obese subjects were oxidizing their body protein and were forced to the conclusion that they were utilizing their own body fat. The latter appeared to be as easily available for heat production as in the normal individual. Likewise we were unable to confirm Hetenyi's contention that there is a difference in the response of the total serum lipids in obese and normal subjects to underfeeding. We can not interpret the variations in blood lipids. We feel that these variations are not associated with any intrinsic abnormality of the adipose tissue cell. The lipemia during fasting in mice found by MacLachlan[6] in contrast to the slight fall of blood lipids found by Chaikoff, *et al.*,[7] in both normal and obese dogs during undernutrition indicates at least one other factor, a species variability.

---

4 Boyd, E. M., *J. Lab. and Clin. Med.*, 1936, **22**, 956.

5 Newburgh, L. H., Johnston, M. W., Lashmet, F. H., and Sheldon, J. M., *J. Nutrition*, 1937, **13**, 203.

6 MacLachlan, P. L., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 411.

7 Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, 1942, **142**, 129.

TABLE I.
Nitrogen Balance, Total Serum Lipids and Weight Loss of Obese Subjects During Undernutrition.

Subject	Diet	Days of diet	*Nitrogen balance, g	Total serum lipids, mg%	Avg 24-hr heat production	†Wt loss (lbs) Actual	Predicted
B.B.	*Ad lib.*			699.9			
Ht. 5′6½	80% basal	7	+1.61	807.7			
Wt. 281	60% "	7	+0.87	868.1	2932	19.75	19.6
	40% "	7	+0.40	910.0			
	20% "	11	—0.37	710.0			
	20% "	7	—0.37	493.0			
L.W.	*Ad lib.*			540.7			
Ht. 5′7¼	80% basal	7	+1.21	574.4			
Wt. 248	60% "	7	+4.62	700.0	2409	17.0	17.1
	40% "	7	+3.59	850.0			
	20% "	7	+1.20	950.0			
	20% "	7	+1.20	630.0			
O.P.	*Ad lib.*			560.0			
Ht. 5′7″	80% basal	7	+1.09	700.0			
Wt. 227	60% "	7	+1.69	856.0	3000	11.0	10.5
	40% "	7	+1.60	610.0			
	20% "	7	+1.70	605.0			

*First three days on each diet omitted.
†Loss for entire experimental period except for first 3 days.

TABLE II.
Nitrogen Balance and Total Serum Lipids of Control Subjects During Undernutrition for 7-day Diet.

Subject	Diet	*Nitrogen balance, g	Total serum lipids, mg%
R.P.	*Ad lib.*		523
Ht. 5′5″	80% basal	—1.12	706
Wt. 124.5	60% "	+0.08	846
	40% "	—1.40	703
L.A.	*Ad lib.*		610
Ht. 5′4″	80% basal	+1.63	750
Wt. 122½	60% "	+0.76	843
	40% "	+0.26	607
M.M.	*Ad lib.*		499
Ht. 5′3″	80% basal	+0.21	710
Wt. 113	60% "	+2.21	806
	40% "	+0.82	542

*Calculated as in Table I.

Table I also shows the actual weight loss of our obese subjects in comparison to their predicted weight loss. In each instance these values are in close agreement. However, our predicted values for weight loss are calculated on the basis of loss of body fat. Were we to assume that body fat can not be mobilized and oxidized the pre-

dicted values for weight loss would have, of necessity, to be calculated on the basis of loss of body protein. In such a case our predicted values would be over twice the actual values. We feel that the agreement in our values (calculated on the basis of fat loss) is very strong evidence in favor of the view that body fat is actually utilized and adds further evidence against the existence of lipophilia.

Summary. Three obese subjects remained in positive nitrogen balance during a prolonged period of undernutrition. The blood lipids varied in essentially the same manner as in 3 control subjects. The actual weight loss during undernutrition was in very close agreement with that predicted on the assumption that body fat was utilized for heat production. These results indicated that the concept of the existence of lipophilia in human obesity is untenable.

The author wishes to express his appreciation to Dr. L. H. Newburgh for valuable assistance in carrying out this investigation.